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(54) Title: OB PROTEIN DERIVATIVES HAVING PROLONGED HALF-LIFE

(57) Abstract

The present invention concerns long half-life derivative of the obesity protein OB. The invention specifically concerns OB protein-immunoglobulin chimeras and polyethylene glycol (PEG)-OB derivatives, which have extended half-life as compared to the corresponding native OB proteins. The invention further relates to methods for appetite and/or weight reduction and for treating other physiological conditions by using the long half-life derivatives of OB.

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OB PROTEIN DERIVATIVES HAVING PROLONGED HALF-LIFE

Field of the Invention

The invention concerns long half-life derivatives of the OB protein. In particular, the invention concerns OB protein-immunoglobulin chimeras, and other long half-life derivatives of the OB protein, and compositions comprising and methods for administering them. The invention further relates to a method for treating obesity by administering a long half-life variant of the OB protein, such as, an OB protein-immunoglobulin chimera.

Background of the Invention

Obesity is the most common nutritional disorder which, according to recent epidemiologic studies, affects about one third of all Americans 20 years of age or older. Kuczmarski *et al.*, J. Am. Med. Assoc. 272, 205-11 (1994). Obesity is responsible for a variety of serious health problems, including cardiovascular disorders, type II diabetes, insulin-resistance, hypertension, hypertriglyceridemia, dyslipoproteinemia, and some forms of cancer. Pi-Sunyer, F.X., Anns. Int. Med. 119, 655-60 (1993); Colfitz, G.A., Am. J. Clin. Nutr. 55, 503S-507S (1992). A single-gene mutation (the obesity or "*ob*" mutation) has been shown to result in obesity and type II diabetes in mice. Friedman, Genomics 11, 1054-1062 (1991). Zhang *et al.*, Nature 372, 425-431 (1994) have recently reported the cloning and sequencing of the mouse *ob* gene and its human homologue, and suggested that the *ob* gene product may function as part of a signalling pathway from adipose tissue that acts to regulate the size of the body fat depot. Parabiosis experiments performed more than 20 years ago predicted that the genetically obese mouse containing two mutant copies of the *ob* gene (*ob/ob* mouse) does not produce a satiety factor which regulates its food intake, while the diabetic (*db/db*) mouse produces but does not respond to a satiety factor. Coleman and Hummal, Am. J. Physiol. 217, 1298-1304 (1969); Coleman, Diabetol 9, 294-98 (1973). Recent reports by three independent research teams have demonstrated that daily injections of recombinant OB protein inhibit food intake and reduce body weight and fat in grossly obese *ob/ob* mice but not in *db/db* mice (Pelleymounter *et al.*, Science 269, 540-43 [1995]; Halaas *et al.*, Science 269, 543-46 [1995]; Campfield *et al.*, Science 269, 546-49 [1995]), suggesting that the *ob* protein is such a satiety factor as proposed in early cross-circulation studies. The results of these first studies leave many questions unanswered, and show a number of as yet unresolved discrepancies. For example, while modest effects of daily injections of the *ob* protein on food intake and body weight were reported in lean mice, there was a significant reduction in body fat as assessed by carcass composition in one (Halaas *et al.*, *supra*) but not in another (Pelleymounter *et al.*, *supra*) of these reports, despite equivalent decreases in body weight. Furthermore, Pelleymounter *et al.*, *supra* observed that, for reasons unknown, *ob/ob* mice treated with a 0.1 mg/kg/day dose of the OB protein actually increased their body weight by 17.13 %, while the weight reduction in the obese mice that received a 1 mg/kg/day dose of *ob* was rather moderate. The receptor or receptors of the *ob* protein are as of yet unidentified. While the existence of peripheral receptors cannot be ruled out at this time, the recent report that an increased

expression of the *ob* gene in adipose tissue of mice with hypothalamic lesions does not result in a lean phenotype suggests that the OB protein does not act directly on fat cells. Maffei *et al.*, Proc. Natl. Acad. Sci. 92, 6957-60 (1995). Researchers suggest that at least one OB receptor is localized in the brain. The identification and expression cloning of a leptin receptor (OB-R) was reported by Tartaglia *et al.*, Cell 83, 1263-71 (1995). Various isoforms of a leptin receptor are described by Cioffi *et al.*, Nature 2, 585-89 (1996). A human hematopoetin receptor, which might be a receptor of the OB protein, is described in PCT application Publication No. WO 96/08510, published 21 March 1996. A receptor of the OB protein is disclosed in Tartaglia *et al.*, Cell 83, 1263-71 (1995).

Summary of the Invention

The present invention is based on the observation that the OB protein is significantly more effective at reducing body weight and adipose tissue weight when delivered as a continuous subcutaneous infusion than when the same dose is delivered as a daily subcutaneous injection. The invention is further based on the unexpected finding that a chimeric protein, in which the OB polypeptide is fused to an immunoglobulin constant domain, is strikingly more potent in reducing the body weight and adipose depots than native human OB, when both proteins are administered by subcutaneous injection once a day. The latter observation is particularly surprising since the OB protein-immunoglobulin chimera due to its large molecular weight, is not expected to be able to cross the blood-brain barrier, and reach the OB receptor which has been believed to be located in the brain.

In one aspect, the invention concerns long half-life derivatives of an OB protein capable of reducing body weight and/or food intake in an individual treated. The invention further concerns compositions containing such derivatives, and their administration for reducing body weight and/or food intake.

In another aspect, the invention concerns chimeric polypeptides comprising an OB protein amino acid sequence capable of binding to a native OB receptor linked to an immunoglobulin sequence (briefly referred to as OB-immunoglobulin chimeras or immunoadhesins). In a specific embodiment, the chimeric polypeptides comprise a fusion of an OB amino acid sequence capable of binding a native OB receptor, to an immunoglobulin constant domain sequence. The OB portion of the chimeras of the present invention preferably has sufficient amino acid sequences from a native OB protein to retain the ability to bind to and signal through a native OB receptor. Most preferably, the OB protein retains the ability to reduce body weight when administered to obese human or non-human subjects. The OB polypeptide is preferably human, and the fusion is preferably with an immunoglobulin heavy chain constant domain sequence. In a particular embodiment, the association of two OB polypeptide-immunoglobulin heavy chain fusions (e.g., via covalent linkage by disulfide bond(s)) results in a homodimeric immunoglobulin-like structure. An immunoglobulin light chain may further be associated with one or both of the OB-immunoglobulin chimeras in the disulfide-bonded dimer to yield a homotrimeric or homotetrameric structure.

The invention further concerns nucleic acid encoding chimeric polypeptide chains of the present invention, expression vectors containing DNA encoding such molecules, transformed host cells, and methods for the production of the molecules by cultivating transformant host cells.

Although the long half-life derivatives of the present invention are particularly useful for reducing body weight and/or food intake, they can generally be used for the treatment of conditions associated with the abnormal expression or function of the OB gene and/or to elicit biological responses mediated by an OB receptor. Thus, the OB derivatives of the present invention may be used to treat bulemia, to reduce insulin levels, e.g. in Type I or II diabetic patients, and as mitogens of various cell types expressing an OB receptor. All these and related uses are within the scope of the present invention.

In another embodiment, the invention concerns the purification of an OB receptor by using an OB protein-immunoglobulin chimera.

Brief Description of the Figures

Figure 1 top -- Lean female mice were treated with murine OB protein either as a continuous subcutaneous infusion or daily subcutaneous injections. The data shown are the mean body weight of each group, in grams, n = 4 mice/point.

Figure 1 bottom -- The mean weight of the retroperitoneal fat pads are shown. Continuous subcutaneous infusions of the OB protein were also more effective than daily subcutaneous injections at reducing adipose tissue weight.

Figure 2 top -- Obese female *ob/ob* mice were treated with human OB protein (hOB) or with a human OB-IgG-1 fusion protein (hOB-IgG-1). The data shown are the mean change in body weight for each treatment group from the first to the last day of experiment, in grams, n = 3 mice/bar except for the hOB 0.19 mg/kg/day by injection group, where n = 4, and PBS injection group, where n = 1.

Figure 2 bottom -- The data shown were the mean food intake for each treatment group for the six 24 hour periods of the experiment, in grams/mouse/day, n = 1/bar.

Figure 3 top and bottom -- Obese (*ob/ob*) female mice were treated with either hOB or the hOB-IgG-1 fusion protein by daily subcutaneous injections for 7 days. The data are depicted as in Figure 2, with n = 4 for all treatment groups.

Figure 4 top - - Obese female *ob/ob* mice were treated with human protein (hOB) or with PEG-hOB. The data shown are the mean change in body weight for each treatment group from the first to the last day of experiment, in grams, n = 3-4 mice/bar except for the PBS injection group, where n = 1. The materials were injected daily subcutaneously. The "PEG 1X" and "PEG 2X" refer to the ratio of the PEG reagent to protein in the preparation of the molecule.

Figure 4 bottom - - The data shown were the mean food intake for each treatment group for the six 24 hour periods of the experiment, in grams/mouse/day, n = 3-4/bar.

Figure 5 - - Obese (*ob/ob*) female mice were treated with either the hOB-IgG fusion protein, native hOB, or hCD4-IgG by daily subcutaneous injections for 7 days. n = 6 for all treatment groups, except hOB at 3.8 mg/kg/d, where n = 2. Again it was observed that the fusion protein was more effective than the native hOB protein at reducing body weight (top and middle panels) and food intake (bottom panel).

Figure 6 - - The nucleotide sequence (SEQ. ID. NO:1) and the amino acid sequence (SEQ. ID. NO: 2) of the human OB-IgG-1 chimera of Example 1.

Detailed Description of the Invention

A. Definitions

The term "obesity" is used to designate a condition of being overweight associated with excessive bodily fat. The desirable weight for a certain individual depends on a number of factors including sex, height, age, overall built, etc. The same factors will determine when an individual is considered obese. The determination of an optimum body weight for a given individual is well within the skill of an ordinary physician.

The phrase "long half-life" and grammatical variants thereof, as used in connection with OB derivatives, concerns OB derivatives having a longer plasma half-life and/or slower clearance than a corresponding native OB protein. The long half-life derivatives preferably will have a half-life at least about 1.5-times longer than a native OB protein; more preferably at least about 2-times longer than a native OB protein, more preferably at least about 3-time longer than a native OB protein. The native OB protein preferably is that of the individual to be treated.

The terms "OB", "OB polypeptide" , "OB protein" and their grammatical variants are used interchangeably and refer to "native" or "native sequence" OB proteins (also known as "leptins") and their functional derivatives. The OB polypeptides have the typical structural features of cytokines, i.e. polypeptides released by one cell population which act on another cell as intercellular mediators, such as, for example, growth hormones, insulin-like growth factors, interleukins, insulin, glycoprotein hormones such as, follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), tumor necrosis factor-α and -β (TNF-α and -β), nerve growth factors, such as NGF-β, PDGF, transforming growth factors (TGFs) such as, TGF-α and TGF-β, insulin-like growth factor-1 and -2 (IGF-1 and IGF-2), erythropoietin, osteoinductive factors, interferons (IFNs) such as, IFN-α, IFN-β and IFN-γ, colony stimulating factors (CSFs) such as, M-CSF, GM-CSF, and G-CSF, interleukins (ILs) such as, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8 and other polypeptide factors.

The terms "native" and "native sequence" OB polypeptide are used to refer to an OB polypeptide from any animal species (e.g. human, murine, rabbit, cat, cow, sheep, chicken, porcine, equine, etc.), as occurring in nature, including naturally-occurring alleles, deletion, substitution and/or insertion variants, as currently known or as might be identified in the future, provided that they retain the ability to bind to and, preferably, signal through the OB receptor. Thus, a native human OB polypeptide includes the amino acid sequence between the N-terminus and the cysteine (Cys) at position 167 of the amino acid sequence shown in Figure 6 (see also SEQ. ID. NO: 2 and Figure 6 of Zhang *et al.*, *supra*), and naturally occurring variants of this protein, as currently known or might be identified in the future. Similarly, a "native" or "native sequence" murine OB polypeptide has the amino acid sequence shown in Figure 6 of Zhang *et al.*, *supra*, and naturally occurring variants of that polypeptide, as currently known or might be identified in the future. The definition specifically includes variants with or without a glutamine at amino acid position 49, using the amino acid numbering of Zhang *et al.*, *supra*. The terms "native" and "native sequence" OB polypeptide include the native proteins with or without the initiating N-terminal methionine (Met), and with or without the native signal sequence, either in monomeric or in dimeric form. The native human and murine OB polypeptides known in the art are 167 amino acids long, contain two conserved cysteines, and have the features of a secreted protein. The polypeptide is largely

hydrophilic, and the predicted signal sequence cleavage site is at position 21, using the amino acid numbering of Zhang *et al.*, *supra*. The overall sequence homology of the human and murine sequences is about 84%. The two proteins show a more extensive identity in the N-terminal region of the mature protein, with only four conservative and three non-conservative substitutions among the residues between the signal sequence cleavage site and the conserved Cys at position 117. The molecular weight of OB proteins is about 16 kD in a monomeric form.

A "functional derivative" of a native polypeptide is a compound having a qualitative biological property in common with the native polypeptide. A functional derivative of an OB polypeptide is a compound that has a qualitative biological property in common with a native (human or non-human) OB polypeptide. "Functional derivatives" include, but are not limited to, fragments of native polypeptides from any animal species (including humans), and derivatives of native (human and non-human) polypeptides and their fragments, provided that they have a biological activity in common with a corresponding native polypeptide.

"Fragments" comprise regions within the sequence of a mature native OB polypeptide. Preferred fragments of OB polypeptides include the C-terminus of the mature protein, and may contain relatively short deletion(s) at the N-terminus and in other parts of the molecule not required for receptor binding and/or for structural integrity.

The term "derivative" is used to define amino acid sequence variants, and covalent modifications of a native polypeptide, whereas the term "variant" refers to amino acid sequence variants within this definition.

"Biological property" in the context of the definition of "functional derivatives" is defined as either 1) immunological cross-reactivity with at least one epitope of a native polypeptide (e.g. a native OB polypeptide of any species), or 2) the possession of at least one adhesive, regulatory or effector function qualitatively in common with a native polypeptide.

Preferably, the functional derivatives are polypeptides which have at least about 65% amino acid sequence identity, more preferably about 75% amino acid sequence identity, even more preferably at least about 85% amino acid sequence identity, most preferably at least about 95% amino acid sequence identity with a native polypeptide. In the context of the present invention, functional derivatives of native sequence human OB polypeptides preferably show at least 95% amino acid sequence identity with the native OB proteins, and are not immunogenic in the human.

Amino acid sequence identity or homology is defined herein as the percentage of amino acid residues in the candidate sequence that are identical with the residues of a corresponding native polypeptide sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent homology, and not considering any conservative substitutions as part of the sequence identity. Neither N- or C-terminal extensions nor insertions shall be construed as reducing identity or homology.

Immunologically cross-reactive as used herein means that the candidate (poly)peptide is capable of competitively inhibiting the qualitative biological activity of a corresponding native polypeptide having this activity with polyclonal antibodies or antisera raised against the known active molecule. Such antibodies and antisera are prepared in conventional fashion by injecting an animal such as a goat or rabbit, for example,

subcutaneously with the known native OB protein in complete Freud's adjuvant, followed by booster intraperitoneal or subcutaneous injection in incomplete Freud's.

The term "isolated OB polypeptide" and grammatical variants thereof refer to OB polypeptides (as hereinabove defined) separated from contaminant polypeptides present in the human, other animal species, or in other source from which the polypeptide is isolated.

In general, the term "amino acid sequence variant" refers to molecules with some differences in their amino acid sequences as compared to a reference (e.g. native sequence) polypeptide. The amino acid alterations may be substitutions, insertions, deletions or any desired combinations of such changes in a native amino acid sequence.

Substitutional variants are those that have at least one amino acid residue in a native sequence removed and a different amino acid inserted in its place at the same position. The substitutions may be single, where only one amino acid in the molecule has been substituted, or they may be multiple, where two or more amino acids have been substituted in the same molecule.

Insertional variants are those with one or more amino acids inserted immediately adjacent to an amino acid at a particular position in a native amino acid sequence. Immediately adjacent to an amino acid means connected to either the α-carboxy or α-amino functional group of the amino acid.

Deletional variants are those with one or more amino acids in the native amino acid sequence removed. Ordinarily, deletional variants will have one or two amino acids deleted in a particular region of the molecule.

"Covalent derivatives" include modifications of a native polypeptide or a fragment thereof with an organic proteinaceous or non-proteinaceous derivatizing agent, and post-translational modifications. Covalent modifications are traditionally introduced by reacting targeted amino acid residues with an organic derivatizing agent that is capable of reacting with selected sites or terminal residues, or by harnessing mechanisms of post-translational modifications that function in selected recombinant host cells. Certain post-translational modifications are the result of the action of recombinant host cells on the expressed polypeptide. Glutaminyl and asparaginyl residues are frequently post-translationally deamidated to the corresponding glutamyl and aspartyl residues. Alternatively, these residues are deamidated under mildly acidic conditions. Either form of these residues may be present in the OB-immunoglobulin chimeras of the present invention. Other post-translational modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl, tyrosine or threonyl residues, methylation of the α-amino groups of lysine, arginine, and histidine side chains [T.E. Creighton, Proteins: Structure and Molecular Properties, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)].

The terms "DNA sequence encoding", "DNA encoding" and "nucleic acid encoding" refer to the order or sequence of deoxyribonucleotides along a strand of deoxyribonucleic acid. The order of these deoxyribonucleotides determines the order of amino acids along the polypeptide chain. The DNA sequence thus codes for the amino acid sequence.

The terms "replicable expression vector" and "expression vector" refer to a piece of DNA, usually double-stranded, which may have inserted into it a piece of foreign DNA. Foreign DNA is defined as heterologous DNA, which is DNA not naturally found in the host cell. The vector is used to transport the

foreign or heterologous DNA into a suitable host cell. Once in the host cell, the vector can replicate independently of the host chromosomal DNA, and several copies of the vector and its inserted (foreign) DNA may be generated. In addition, the vector contains the necessary elements that permit translating the foreign DNA into a polypeptide. Many molecules of the polypeptide encoded by the foreign DNA can thus be rapidly synthesized.

The term "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, a ribosome binding site, and possibly, other as yet poorly understood sequences. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancer.

Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or a secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, then synthetic oligonucleotide adaptors or linkers are used in accord with conventional practice.

In the context of the present invention the expressions "cell", "cell line", and "cell culture" are used interchangeably, and all such designations include progeny. Thus, the words "transformants" and "transformed (host) cells" include the primary subject cell and cultures derived therefrom without regard for the number of transfers. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Mutant progeny that have the same function or biological activity as screened for in the originally transformed cell are included. Where distinct designations are intended, it will be clear from the context.

Native immunoglobulins are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies between the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain (V_H) followed by a number of constant domains. Each light chain has a variable domain at one and (V_L) and a constant domain at its other end; the constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light and heavy chain variable domains (Clothia *et al.*, J. Mol. Biol. 186, 651-663 (1985); Novotny and Haber, Proc. Natl. Acad. Sci. USA 82, 4592-4596 [1985]).

Depending on the amino acid sequence of the constant region of their heavy chains, immunoglobulins can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG and

IgM, and several of these may be further divided into subclasses (isotypes), e.g. IgG-1, IgG-2, IgG-3, and IgG-4; IgA-1 and IgA-2. The heavy chain constant regions that correspond to the different classes of immunoglobulins are called α, delta, epsilon, γ, and μ, respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known. IgA-1 and IgA-2 are monomeric subclasses of IgA, which usually is in the form of dimers or larger polymers. Immunocytes in the gut produce mainly polymeric IgA (also referred to poly-IgA including dimers and higher polymers). Such poly-IgA contains a disulfide-linked polypeptide called the "joining" or "J" chain, and can be transported through the glandular epithelium together with the J-containing polymeric IgM (poly-IgM), comprising five subunits.

Hybridization is preferably performed under "stringent conditions" which means (1) employing low ionic strength and high temperature for washing, for example, 0.015 sodium chloride/0.0015 M sodium citrate/0.1% sodium dodecyl sulfate at 50°C, or (2) employing during hybridization a denaturing agent, such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50nM sodium phosphate buffer at pH 6.5 with 750 mM sodium chloride, 75 mM sodium citrate at 42°C. Another example is use of 50% formamide, 5 x SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6/8), 0.1% sodium pyrophosphate, 5 x Denhardt's solution, sonicated salmon sperm DNA (50 μg/ml), 0.1% SDS, and 10% dextran sulfate at 42°C, with washes at 42°C in 0.2 x SSC and 0.1% SDS.

B. OB protein-immunoglobulin chimeras (immunoadhesins)

Immunoadhesins are chimeric antibody-like molecules that combine the functional domain(s) of a binding protein (usually a receptor, a cell-adhesion molecule or a ligand) with the an immunoglobulin sequence. The most common example of this type of fusion protein combines the hinge and Fc regions of an immunoglobulin (Ig) with domains of a cell-surface receptor that recognizes a specific ligand. This type of molecule is called an "immunoadhesin", because it combines "immune" and "adhesion" functions; other frequently used names are "Ig-chimera", "Ig-" or "Fc-fusion protein", or "receptor-globulin."

To date, more than fifty immunoadhesins have been reported in the art. Immunoadhesins reported in the literature include, for example, fusions of the T cell receptor (Gascoigne *et al.*, Proc. Natl. Acad. Sci. USA 84, 2936-2940 [1987]); CD4 (Capon *et al.*, Nature 337, 525-531 [1989]; Traunecker *et al.*, Nature 339, 68-70 [1989]; Zettmeissl *et al.*, DNA Cell Biol. USA 9, 347-353 [1990]; Byrn *et al.*, Nature 344, 667-670 [1990]); L-selectin (homing receptor) (Watson *et al.*, J. Cell. Biol. 110, 2221-2229 [1990]; Watson *et al.*, Nature 349, 164-167 [1991]); E-selectin [Mulligan *et al.*, J. Immunol. 151, 6410-17 [1993]; Jacob *et al.*, Biochemistry 34, 1210-1217 [1995]); P-selectin (Mulligan *et al.*, *supra*; Hollenbaugh *et al.*, Biochemistry 34, 5678-84 [1995]); ICAM-1 (Stauton *et al.*, J. Exp. Med. 176, 1471-1476 [1992]; Martin *et al.*, J. Virol. 67, 3561-68 [1993]; Roep *et al.*, Lancet 343, 1590-93 [1994]); ICAM-2 (Damle *et al.*, J. Immunol. 148, 665-71 [1992]); ICAM-3 (Holness *et al.*, J. Biol. Chem. 270, 877-84 [1995]); LFA-3 (Kanner *et al.*, J. Immunol. 148, 2-23-29 [1992]); L1 glycoprotein (Doherty *et al.*, Neuron 14, 57-66 [1995]); TNF-R1 (Ashkenazi *et al.*, Proc. Natl. Acad. Sci. USA 88, 10535-539 [1991]; Lesslauer *et al.*, Eur. J. Immunol. 21, 2883-86 [1991]; Peppel *et al.*, J. Exp. Med. 174, 1483-1489 [1991]); TNF-R2 (Zack *et al.*, Proc. Natl. Acad. Sci. USA 90, 2335-39 [1993]; Wooley *et al.*, J.

Immunol. 151, 6602-07 [1993]); CD44 [Aruffo *et al.*, Cell 61, 1303-1313 (1990)]; CD28 and B7 [Linsley *et al.*, J. Exp. Med. 173, 721-730 (1991)]; CTLA-4 [Lisley *et al.*, J. Exp. Med. 174, 561-569 (1991)]; CD22 [Stamenkovic *et al.*, Cell 66. 1133-1144 (1991)]; NP receptors [Bennett *et al.*, J. Biol. Chem. 266, 23060-23067 (1991)]; IgE receptor α [Ridgway and Gorman, J. Cell. Biol. 115, abstr. 1448 (1991)]; HGF receptor [Mark, M.R. *et al.*, 1992, J. Biol. Chem. submitted]; IFN-γR α- and β-chain [Marsters *et al.*, Proc. Natl. Acad. Sci. USA 92, 5401-05 [1995]); trk-A, -B, and -C (Shelton *et al.*, J. Neurosci. 15, 477-91 [1995]); IL-2 (Landolfi. J. Immunol. 146, 915-19 [1991]); IL-10 (Zheng *et al.*, J. Immunol. 154, 5590-5600 [1995]).

The simplest and most straightforward immunoadhesin design combines the binding region(s) of the 'adhesin' protein with the hinge and Fc regions of an immunoglobulin heavy chain. Ordinarily, when preparing the OB-immunoglobulin chimeras of the present invention, nucleic acid encoding the desired OB polypeptide will be fused C-terminally to nucleic acid encoding the N-terminus of an immunoglobulin constant domain sequence, however N-terminal fusions are also possible. Typically, in such fusions the encoded chimeric polypeptide will retain at least functionally active hinge, CH2 and CH3 domains of the constant region of an immunoglobulin heavy chain. Fusions are also made to the C-terminus of the Fc portion of a constant domain, or immediately N-terminal to the CH1 of the heavy chain or the corresponding region of the light chain. The precise site at which the fusion is made is not critical; particular sites are well known and may be selected in order to optimize the biological activity, secretion or binding characteristics of the OB-immunoglobulin chimeras.

In a preferred embodiment, the sequence of a native, mature OB polypeptide, is fused to the N-terminus of the C-terminal portion of an antibody (in particular the Fc domain), containing the effector functions of an immunoglobulin, e.g. IgG-1. It is possible to fuse the entire heavy chain constant region to the OB sequence. However, more preferably, a sequence beginning in the hinge region just upstream of the papain cleavage site (which defines IgG Fc chemically; residue 216, taking the first residue of heavy chain constant region to be 114 [Kobet *et al.*, *supra*], or analogous sites of other immunoglobulins) is used in the fusion. In a particularly preferred embodiment, the OB polypeptide sequence is fused to the hinge region and CH2 and CH3 or CH1, hinge, CH2 and CH3 domains of an IgG-1, IgG-2, or IgG-3 heavy chain. The precise site at which the fusion is made is not critical, and the optimal site can be determined by routine experimentation.

In some embodiments, the OB-immunoglobulin chimeras are assembled as multimers, and particularly as homo-dimers or -tetramers (WO 91/08298). Generally, these assembled immunoglobulins will have known unit structures. A basic four chain structural unit is the form in which IgG, IgD, and IgE exist. A four unit is repeated in the higher molecular weight immunoglobulins; IgM generally exists as a pentamer of basic four units held together by disulfide bonds. IgA globulin, and occasionally IgG globulin, may also exist in multimeric form in serum. In the case of multimer, each four unit may be the same or different.

Various exemplary assembled OB-immunoglobulin chimeras within the scope herein are schematically diagrammed below:

(a) AC_L-AC_L;

(b) AC_H-[AC_H, AC_L-AC_H, AC_L-V_HC_H, or V_LC_L-AC_H];

(c) AC_L-AC_H-[AC_L-AC_H, AC_L-V_HC_H, V_LC_L-AC_H, or V_LC_L-V_HC_H];

(d) AC_L-V_HC_H-[AC_H, or AC_L-V_HC_H, or V_LC_L-AC_H];

(e) V_LC_L-AC_H-[AC_L-V_HC_H, or V_LC_L-AC_H]; and

(f) $[A\text{-}Y]_n$-$[V_LC_L\text{-}V_HC_H]_2$,

wherein

each A represents identical or different OB polypeptide amino acid sequences;

V_L is an immunoglobulin light chain variable domain;

V_H is an immunoglobulin heavy chain variable domain;

C_L is an immunoglobulin light chain constant domain;

C_H is an immunoglobulin heavy chain constant domain;

n is an integer greater than 1;

Y designates the residue of a covalent cross-linking agent.

In the interests of brevity, the foregoing structures only show key features; they do not indicate joining (J) or other domains of the immunoglobulins, nor are disulfide bonds shown. However, where such domains are required for binding activity, they shall be constructed as being present in the ordinary locations which they occupy in the immunoglobulin molecules.

Alternatively, the OB amino acid sequences can be inserted between immunoglobulin heavy chain and light chain sequences such that an immunoglobulin comprising a chimeric heavy chain is obtained. In this embodiment, the OB polypeptide sequences are fused to the 3' end of an immunoglobulin heavy chain in each arm of an immunoglobulin, either between the hinge and the CH2 domain, or between the CH2 and CH3 domains. Similar constructs have been reported by Hoogenboom, H. R. *et al.*, Mol. Immunol. 28, 1027-1037 (1991).

Although the presence of an immunoglobulin light chain is not required in the immunoadhesins of the present invention, an immunoglobulin light chain might be present either covalently associated to an OB protein-immunoglobulin heavy chain fusion polypeptide, or directly fused to the OB polypeptide. In the former case, DNA encoding an immunoglobulin light chain is typically coexpressed with the DNA encoding the OB-immunoglobulin heavy chain fusion protein. Upon secretion, the hybrid heavy chain and the light chain will be covalently associated to provide an immunoglobulin-like structure comprising two disulfide-linked immunoglobulin heavy chain-light chain pairs. Method suitable for the preparation of such structures are, for example, disclosed in U.S. Patent No. 4,816,567 issued 28 March 1989.

In a preferred embodiment, the immunoglobulin sequences used in the construction of the immunoadhesins of the present invention are from an IgG immunoglobulin heavy chain constant domain. For human immunoadhesins, the use of human IgG-1 and IgG-3 immunoglobulin sequences is preferred. A major advantage of using IgG-1 is that IgG-1 immunoadhesins can be purified efficiently on immobilized protein A. In contrast, purification of IgG-3 requires protein G, a significantly less versatile medium. However, other structural and functional properties of immunoglobulins should be considered when choosing the Ig fusion partner for a particular immunoadhesin construction. For example, the IgG-3 hinge is longer and more flexible, so it can accommodate larger 'adhesin' domains that may not fold or function properly when fused to IgG-1. Possible IgG-based immunoadhesin structures are shown in Fig. 3a-c. While IgG immunoadhesins are typically

mono- or bivalent, other Ig subtypes like IgA and IgM may give rise to dimeric or pentameric structures, respectively, of the basic Ig homodimer unit. A typical IgM-based multimeric immunoadhesin is illustrated in Figure 3d. Multimeric immunoadhesins are advantageous in that they can bind their respective targets with greater avidity than their IgG-based counterparts. Reported examples of such structures are CD4-IgM (Traunecker *et al.*, *supra*); ICAM-IgM (Martin *et al.*, J. Virol. 67, 3561-68 [1993]); and CD2-IgM (Arulanandam *et al.*, J. Exp. Med. 177, 1439-50 [1993]).

For OB-Ig immunoadhesins, which are designed for *in vivo* application, the pharmacokinetic properties and the effector functions specified by the Fc region are important as well. Although IgG-1, IgG-2 and IgG-4 all have *in vivo* half-lives of 21 days, their relative potencies at activating the complement system are different. IgG-4 does not activate complement, and IgG-2 is significantly weaker at complement activation than IgG-1. Moreover, unlike IgG-1, IgG-2 does not bind to Fc receptors on mononuclear cells or neutrophils. While IgG-3 is optimal for complement activation, its *in vivo* half-life is approximately one third of the other IgG isotypes. Another important consideration for immunoadhesins designed to be used as human therapeutics is the number of allotypic variants of the particular isotype. In general, IgG isotypes with fewer serologically-defined allotypes are preferred. For example, IgG-1 has only four serologically-defined allotypic sites, two of which (G1m and 2) are located in the Fc region; and one of these sites G1m1, is non-immunogenic. In contrast, there are 12 serologically-defined allotypes in IgG-3, all of which are in the Fc region; only three of these sites (G3m5, 11 and 21) have one allotype which is nonimmunogenic. Thus, the potential immunogenicity of a γ3 immunoadhesin is greater than that of a γ1 immunoadhesin.

In designing the OB-Ig immunoadhesins of the present invention regions that are not required for receptor binding, the structural integrity (e.g. proper folding) and/or biological activity of the molecule, may be deleted. In such structures, it is important to place the fusion junction at residues that are located between domains, to avoid misfolding. With respect to the parental immunoglobulin, a useful joining point is just upstream of the cysteines of the hinge that form the disulfide bonds between the two heavy chains. In a frequently used design, the codon for the C-terminal residue of the "adhesin" (OB) part of the molecule is placed directly upstream of the codons for the sequence DKTHTCPPCP of the IgG1 hinge region.

OB-Ig immunoadhesins are most conveniently constructed by fusing the cDNA sequence encoding the OB portion in-frame to an Ig cDNA sequence. However, fusion to genomic Ig fragments can also be used (see, e.g. Gascoigne *et al.*, Proc. Natl. Acad. Sci. USA 84, 2936-2940 [1987]; Aruffo *et al.*, Cell 61, 1303-1313 [1990]; Stamenkovic *et al.*, Cell 66, 1133-1144 [1991]). The latter type of fusion requires the presence of Ig regulatory sequences for expression. cDNAs encoding IgG heavy-chain constant regions can be isolated based on published sequence from cDNA libraries derived from spleen or peripheral blood lymphocytes, by hybridization or by polymerase chain reaction (PCR) techniques. Murine OB cDNA can, for example, be obtained by PCR from a mouse adipose tissue cDNA library (Clontech), using primers designed based on the sequence of Zhang *et al.* Human OB cDNA can be obtained in a similar manner. Alternatively, the mouse OB gene can be used as a probe to isolate human adipose tissue cDNA clones (Clontech), e.g. from a λgtII library, as described by Zhang *et al.* The cDNAs encoding the 'adhesin' and the Ig parts of the immunoadhesin are inserted in tandem into a plasmid vector that directs efficient expression in the chosen host cells. For

expression in mammalian cells pRK5-based vectors (Schall *et al.*, Cell 61, 361-370 [1990]), pRK7-vectors and CDM8-based vectors (Seed, Nature 329, 840 [1989]) are preferred. (pRK7 is identical to pRK5 except that the order of the endonuclease restriction sites in the polylinker region between ClaI and HindIII is reversed. See U.S. Patent No. 5,108,901 issued 28 April 1992.). The exact junction can be created by removing the extra sequences between the designed junction codons using oligonucleotide-directed deletional mutagenesis (Zoller and Smith, Nucleic Acids Res. 10, 6487 [1982]; Capon *et al.*, Nature 337, 525-531 [1989]). Synthetic oligonucleotides can be used, in which each half is complementary to the sequence on either side of the desired junction; ideally, these are 36 to 48-mers. Alternatively, PCR technique can be used to join the two parts of the molecule in-frame with an appropriate vector.

Immunoadhesins can be expressed efficiently in a variety of host cells, including myeloma cell lines, Chinese Hamster ovary (CHO) cells, monkey COS cells, human embryonic kidney 293 cells, and baculovirus infected insect cells. In these systems, the immunoadhesin polypeptides are assembled and secreted into the cell culture medium. Yeasts, e.g. Saccharomyces cerevisiae, Pichia pastoris, etc., and bacterial cells, preferably E. coli, can also be used as hosts. The OB-immunoglobulin chimeras can be expressed in yeast, for example, similarly to the process described for the expression of the OB proteins by Leiber *et al.*, Crit. Res. Food Sci. Nutr. 33, 351 (1993); Friedman and Leibel, Cell 69, 217 (1992); and Beavis and Chait, Proc. Natl. Acad. Sci. USA 87, 6873 (1990). Thus, the coding sequences can be subcloned into a yeast plasmid, such as the yeast expression plasmid pPIC.9 (Invitrogen). This vector directs secretion of heterologous proteins from the yeast into the culture media. According to Halaas *et al.*, *supra*, expression of mouse and human OB genes in Saccharomyces cerevisiae transformed with this vector yields a secreted 16-kD protein, which is an unprocessed OB protein lacking the signal sequence. Expression of the mouse or human OB-immunoglobulin chimeras in E. coli can, for example, be performed on the analogy of the procedure described by Halaas *et al.*, *supra*. The coding sequences of mouse and human OB-immunoglobulin chimeras can be subcloned into the PET15b expression vector (Novagen) and expressed in E. coli (BL21 (DE3)pIYsS) through use of the T7 E. coli RNA polymerase system. Alternatively, the fusion protein can be expressed in E. coli by inserting the coding sequence in frame with the secretion sequence of the E. coli heat stable enterotoxin II, downstream of the E. coli alkaline phosphatase promoter (Chang *et al.*, Gene 55, 189-96 [1987]).

The choice of host cell line for the expression of OB-Ig immunoadhesins depends mainly on the expression vector. Another consideration is the amount of protein that is required. Milligram quantities often can be produced by transient transfections. For example, the adenovirus EIA-transformed 293 human embryonic kidney cell line can be transfected transiently with pRK5- and pRK7-based vectors by a modification of the calcium phosphate method to allow efficient immunoadhesin expression. This method is illustrated in the examples. CDM8-based vectors can be used to transfect COS cells by the DEAE-dextran method (Aruffo *et al.*, Cell 61, 1303-1313 (1990); Zettmeissl *et al.*, DNA Cell Biol. (US) 9, 347-353 (1990)]. If larger amounts of protein are desired, the immunoadhesin can be expressed after stable transfection of a host cell line. For example, a pRK5- or pRK7-based vector can be introduced into Chinese hamster ovary (CHO) cells in the presence of an additional plasmid encoding dihydrofolate reductase (DHFR) and conferring resistance to G418. Clones resistant to G418 can be selected in culture; these clones are grown in the presence of increasing levels

of DHFR inhibitor methotrexate; clones are selected, in which the number of gene copies encoding the DHFR and immunoadhesin sequences is co-amplified. If the immunoadhesin contains a hydrophobic leader sequence at its N-terminus, it is likely to be processed and secreted by the transfected cells. The expression of immunoadhesins with more complex structures may require uniquely suited host cells; for example, components such as light chain or J chain may be provided by certain myeloma or hybridoma cell hosts [Gascoigne *et al.*, 1987, *supra*; Martin *et al.*, J. Virol. 67, 3561-3568 (1993)].

The expression of immunoadhesins with more complex oligomeric structures may require uniquely suited host cells; for example, components such as light chain or J chain may be provided by certain myeloma or hybridoma cell hosts (Gascoigne *et al.*, *supra*; Martin *et al.*, J. Immunol. 67, 3561-68 [1993]).

Immunoadhesins can be conveniently purified by affinity chromatography. The suitability of protein A as an affinity ligand depends on the species and isotype of the immunoglobulin Fc domain that is used in the chimera. Protein A can be used to purify immunoadhesins that are based on human γ1, γ2, or γ4 heavy chains [Lindmark *et al.*, J. Immunol. Meth. 62, 1-13 (1983)]. Protein G is recommended for all mouse isotypes and for human γ3 [Guss *et al.*, EMBO J. 5, 15671575 (1986)]. The matrix to which the affinity ligand is attached is most often agarose, but other matrices are available. Mechanically stable matrices such as controlled pore glass or poly(styrenedivinyl)benzene allow for faster flow rates and shorter processing times than can be achieved with agarose. The conditions for binding an immunoadhesin to the protein A or G affinity column are dictated entirely by the characteristics of the Fc domain; that is, its species and isotype. Generally, when the proper ligand is chosen, efficient binding occurs directly from unconditioned culture fluid. One distinguishing feature of immunoadhesins is that, for human γ1 molecules, the binding capacity for protein A is somewhat diminished relative to an antibody of the same Fc type. Bound immunoadhesin can be efficiently eluted either at acidic pH (at or above 3.0), or in a neutral pH buffer containing a mildly chaotropic salt. This affinity chromatography step can result in an immunoadhesin preparation that is >95% pure.

Other methods known in the art can be used in place of, or in addition to, affinity chromatography on protein A or G to purify immunoadhesins. Immunoadhesins behave similarly to antibodies in thiophilic gel chromatography [Hutchens and Porath, Anal. Biochem. 159, 217-226 (1986)] and immobilized metal chelate chromatography [Al-Mashikhi and Makai, J. Dairy Sci. 71, 1756-1763 (1988)]. In contrast to antibodies, however, their behavior on ion exchange columns is dictated not only by their isoelectric points, but also by a charge dipole that may exist in the molecules due to their chimeric nature. Microheterogeneity of charge can also be a factor for immunoadhesins in which the adhesin portion of the molecule is glycosylated and contains sialic acid. A specific purification protocol is described in the examples.

Results with the numerous immunoadhesins produced so far show that the fusion of the adhesin portion to an Fc region usually does not perturb the folding of the individual domains. Both the adhesin and the immunoglobulin regions appear to fold correctly, and the Fc portion retins many of the effector functions that are characteristic of antibodies, such as binding to Fc receptors.

Methods generally applicable for the construction, expression and purification of immunoadhesins are described, for example, in U.S. Patent Nos. 5,225,538 (issued 6 July 1993) and 5,455,165 (issued 30 October 1995), the disclosures of which are hereby expressly incorporated by reference. Immunoadhesin construction,

expression, purification and various immunoadhesins designs are also described in the review articles by Ashkenazi and Chamow, Methods in Enzymology 8, 104-115 (1995), and Peach and Linsley, Methods in Enzymology 8, 116-123 (1995), the disclosures of which, along with the references cited therein, is hereby expressly incorporated by reference.

C. **Other long half-life OB derivatives**

Other derivatives of the OB proteins, which possess a longer half-life than the native molecules comprise the OB protein or an OB-immunoglobulin chimera, covalently bonded to a nonproteinaceous polymer. The nonproteinaceous polymer ordinarily is a hydrophilic synthetic polymer, i.e., a polymer not otherwise found in nature. However, polymers which exist in nature and are produced by recombinant or *in vitro* methods are useful, as are polymers which are isolated from native sources. Hydrophilic polyvinyl polymers fall within the scope of this invention, e.g. polyvinylalcohol and polyvinylpyrrolidone. Particularly useful are polyalkylene ethers such as polyethylene glycol (PEG); polyelkylenes such as polyoxyethylene, polyoxypropylene, and block copolymers of polyoxyethylene and polyoxypropylene (Pluronics); polymethacrylates; carbomers; branched or unbranched polysaccharides which comprise the saccharide monomers D-mannose, D- and L-galactose, fucose, fructose, D-xylose, L-arabinose, D-glucuronic acid, sialic acid, D-galacturonic acid, D-mannuronic acid (e.g. polymannuronic acid, or alginic acid), D-glucosamine, D-galactosamine, D-glucose and neuraminic acid including homopolysaccharides and heteropolysaccharides such as lactose, amylopectin, starch, hydroxyethyl starch, amylose, dextrane sulfate, dextran, dextrins, glycogen, or the polysaccharide subunit of acid mucopolysaccharides, e.g. hyaluronic acid; polymers of sugar alcohols such as polysorbitol and polymannitol; heparin or heparon. The polymer prior to cross-linking need not be, but preferably is, water soluble, but the final conjugate must be water soluble. In addition, the polymer should not be highly immunogenic in the conjugate form, nor should it possess viscosity that is incompatible with intravenous infusion or injection if it is intended to be administered by such routes.

Preferably the polymer contains only a single group which is reactive. This helps to avoid cross-linking of protein molecules. However, it is within the scope herein to optimize reaction conditions to reduce cross-linking, or to purify the reaction products through gel filtration or chromatographic sieves to recover substantially homogenous derivatives.

The molecular weight of the polymer may desirably range from about 100 to 500,000, and preferably is from about 1,000 to 20,000. The molecular weight chosen will depend upon the nature of the polymer and the degree of substitution. In general, the greater the hydrophilicity of the polymer and the greater the degree of substitution, the lower the molecular weight that can be employed. Optimal molecular weights will be determined by routine experimentation.

The polymer generally is covalently linked to the OB protein or to the OB-immunoglobulin chimeras though a multifunctional crosslinking agent which reacts with the polymer and one or more amino acid or sugar residues of the OB protein or OB-immunoglobulin chimera to be linked. However, it is within the scope of the invention to directly crosslink the polymer by reacting a derivatized polymer with the hybrid, or via versa.

The covalent crosslinking site on the OB protein or OB-Ig includes the N-terminal amino group and epsilon amino groups found on lysine residues, as well as other amino, imino, carboxyl, sulfhydryl, hydroxyl or other hydrophilic groups. The polymer may be covalently bonded directly to the hybrid without the use of a multifunctional (ordinarily bifunctional) crosslinking agent. Covalent binding to amino groups is accomplished by known chemistries based upon cyanuric chloride, carbonyl diimidazole, aldehyde reactive groups (PEG alkoxide plus diethyl acetal of bromoacetaldehyde; PEG plus DMSO and acetic anhydride, or PEG chloride plus the phenoxide of 4-hydroxybenzaldehyde, succinimidyl active esters, activated dithiocarbonate PEG, 2,4,5-trichlorophenylcloroformate or P-nitrophenylcloroformate activated PEG.) Carboxyl groups are derivatized by coupling PEG-amine using carbodiimide.

Polymers are conjugated to oligosaccharide groups by oxidation using chemicals, e.g. metaperiodate, or enzymes, e.g. glucose or galactose oxidase, (either of which produces the aldehyde derivative of the carbohydrate), followed by reaction with hydrazide or amino derivatized polymers, in the same fashion as is described by Heitzmann *et al.*, P.N.A.S., 71, 3537-41 (1974) or Bayer *et al.*, Methods in Enzymology 62, 310 (1979), for the labeling of oligosaccharides with biotin or avidin. Further, other chemical or enzymatic methods which have been used heretofore to link oligosaccharides are particularly advantageous because, in general, there are fewer substitutions than amino acid sites for derivatization, and the oligosaccharide products thus will be more homogenous. The oligosaccharide substituents also are optionally modified by enzyme digestion to remove sugars, e.g. by neuraminidase digestion, prior to polymer derivatization.

The polymer will bear a group which is directly reactive with an amino acid side chain, or the N- or C-terminus of the polypeptide linked, or which is reactive with the multifunctional cross-linking agent. In general, polymers bearing such reactive groups are known for the preparation of immobilized proteins. In order to use such chemistries here, one should employ a water soluble polymer otherwise derivatized in the same fashion as insoluble polymers heretofore employed for protein immobilization. Cyanogen bromide activation is a particularly useful procedure to employ in crosslinking polysaccharides.

"Water soluble" in reference to the starting polymer means that the polymer or its reactive intermediate used for conjugation is sufficiently water soluble to participate in a derivatization reaction.

"Water soluble" in reference to the polymer conjugate means that the conjugate is soluble in physiological fluids such as blood.

The degree of substitution with such a polymer will vary depending upon the number of reactive sites on the protein, whether all or a fragment of the protein is used, whether the protein is a fusion with a heterologous protein (e.g. an OB-immunoglobulin chimera), the molecular weight, hydrophilicity and other characteristics of the polymer, and the particular protein derivatization sites chosen. In general, the conjugate contains about from 1 to 10 polymer molecules, while any heterologous sequence may be substituted with an essentially unlimited number of polymer molecules so long as the desired activity is not significantly adversely affected. The optimal degree of cross-linking is easily determined by an experimental matrix in which the time, temperature and other reaction conditions are varied to change the degree of substitution, after which the ability of the conjugates to function in the desired fashion is determined.

The polymer, e.g. PEG, is cross-linked by a wide variety of methods known *per se* for the covalent modification of proteins with nonproteinaceous polymers such as PEG. Certain of these methods, however, are not preferred for the purposes herein. Cyanuronic chloride chemistry leads to many side reactions, including protein cross-linking. In addition, it may be particularly likely to lead to inactivation of proteins containing sulfhydryl groups. Carbonyl diimidazole chemistry (Beauchamp *et al.*, Anal Biochem. 131, 25-33 [1983]) requires high pH (>8.5), which can inactivate proteins. Moreover, since the "activated PEG" intermediate can react with water, a very large molar excess of "activated PEG" over protein is required. The high concentrations of PEG required for the carbonyl diimidazole chemistry also led to problems in purification, as both gel filtration chromatography and hydrophilic interaction chromatography are adversely affected. In addition, the high concentrations of "activated PEG" may precipitate protein, a problem that *per se* has been noted previously (Davis, U.S. Patent No. 4,179,337). On the other hand, aldehyde chemistry (Royer, U.S. Patent No. 4,002,531) is more efficient since it requires only a 40-fold molar excess of PEG and a 1-2 hr incubation. However, the manganese dioxide suggested by Royer for preparation of the PEG aldehyde is problematic "because of the pronounced tendency of PEG to form complexes with metal-based oxidizing agents" (Harris *et al.*, J. Polym. Sci. Polym. Chem. Ed. 22, 341-52 [1984]). The use of a Moffatt oxidation, utilizing DMSO and acetic anhydride, obviates this problem. In addition, the sodium borohydride suggested by Royer must be used at high pH and has a significant tendency to reduce disulfide bonds. In contrast, sodium cyanoborohydride, which is effective at neutral pH and has very little tendency to reduce disulfide bonds is preferred.

Functionalized PEG polymers to modify the OB protein or OB-Ig chimeras of the present invention are available from Shearwater Polymers, Inc. (Huntsville, AL). Such commercially available PEG derivatives include, but are not limited to, amino-PEG, PEG amino acid esters, PEG-hydrazide, PEG-thiol, PEG-succinate, carboxymethylated PEG, PEG-propionic acid, PEG amino acids, PEG succinimidyl succinate, PEG succinimidyl propionate, succinimidyl ester of carboxymethylated PEG, succinimidyl carbonate of PEG, succinimidyl esters of amino acid PEGs, PEG-oxycarbonylimidazole, PEG-nitrophenyl carbonate, PEG tresylate, PEG-glycidyl ether, PEG-aldehyde, PEG vinylsulfone, PEG-maleimide, PEG-orthopyridyl-disulfide, heterofunctional PEGs, PEG vinyl derivatives, PEG silanes, and PEG phospholides. The reaction conditions for coupling these PEG derivatives will vary depending on the protein, the desired degree of PEGylation, and the PEG derivative utilized. Some factors involved in the choice of PEG derivatives include: the desired point of attachment (lysine or cysteine), hydrolytic stability and reactivity of the derivatives, stability, toxicity and antigenicity of the linkeage, suitability for analysis, etc. Specific instructions for the use of any particular derivative are available from the manufacturer.

The long half-life conjugates of this invention are separated from the unreacted starting materials by gel filtration. Heterologous species of the conjugates are purified from one another in the same fashion. The polymer also may be water-insoluble, as a hydrophilic gel.

The conjugates may also be purified by ion-exchange chromatography. The chemistry of many of the electrophilically activated PEG's results in a reduction of amino group charge of the PEGylated product. Thus, high resolution ion exchange chromatography can be used to separate the free and conjugated proteins, and to resolve species with different levels of PEGylation. In fact, the resolution of different species (e.g. containing

one or two PEG residues) is also possible due to the difference in the ionic properties of the unreacted amino acids.

D. The use of the OB-immunoglobulin chimeras and other long half-life derivatives

The OB-immunoglobulin chimeras and other long half-life OB derivatives of the present invention are useful for weight reduction, and specifically, in the treatment of obesity and other disorders associated with the abnormal expression or function of the OB gene. Our studies indicate that the OB-immunoglobulin chimeras and other long half-life OB derivatives, e.g. PEGylated OB, reduce the food intake and increase the energy use of animals treated, and are therefore very effective in reducing the weight of both obese and normal subjects. For testing purposes, the molecules of the present invention may be dissolved in phosphate-buffered saline (PBS) (pH 7.4), and administered by intravenous or subcutaneous injection, or infusion.

The long acting OB-derivatives of the present invention may further be used to treat other metabolic disorders such as diabetes and bulimia. The OB protein has been shown to reduce insulin levels in animals, and could be useful to reduce excessive levels of insulin in human patients. The reduction of insulin levels in obese or non-obese patients (e.g. Type I or II diabetics) could restore or improve the insulin-sensitivity of such patients.

In addition, the long half-life OB-derivatives can be used for the treatment of kidney ailments, hypertension, and lung disfunctions, such as emphysema. The OB protein might also cause a mitogenic response in receptor-bearing tissues, acting as a growth factor for these cells.

Therapeutic formulations of the present invention are prepared for storage by mixing the active ingredient having the desired degree of purity with optional physiologically acceptable carriers, excipients or stabilizers (Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed. (1980)), in the form of lyophilized formulations or aqueous solutions. Acceptable carriers, excipients or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate and other organic acids; antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone, amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as Tween, Pluronics or PEG.

The active ingredients may also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacylate) microcapsules, respectively), in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences, *supra*.

The formulations to be used for *in vivo* administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes, prior to or following lyophilization and reconstitution.

Therapeutic compositions herein generally are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle.

The route of administration is in accord with known methods, e.g. injection or infusion by intravenous, intraperitoneal, etc. routes. Sustained released formulations are also foreseen. Suitable examples of sustained release preparations include semipermeable polymer matrices in the form of shaped articles, e.g. films, or microcapsules. Sustained release matrices include polyesters, hydrogels, polylactides (U.S. Patent 3,773,919, EP 58,481), copolymers of L-glutamic acid and gamma ethyl-L-glutamate (U. Sidman et al., 1983, "Biopolymers" 22 (1): 547-556), poly (2-hydroxyethyl-methacrylate) (R. Langer, et al., 1981, "J. Biomed. Mater. Res." 15: 167-277 and R. Langer, 1982, Chem. Tech." 12: 98-105), ethylene vinyl acetate (R. Langer et al., Id.) or poly-D-(-)-3-hydroxybutyric acid (EP 133,988A). Sustained release compositions also include liposomes. Liposomes containing a molecule within the scope of the present invention are prepared by methods known per se: DE 3,218,121A; Epstein et al., 1985, "Proc. Natl. Acad. Sci. USA" 82: 3688-3692; Hwang et al., 1980, "Proc. Natl. Acad. Sci. USA" 77: 4030-4034; EP 52322A; EP 36676A; EP 88046A; EP 143949A; EP 142641A; Japanese patent application 83-118008; U.S. patents 4,485,045 and 4,544,545; and EP 102,324A. Ordinarily the liposomes are of the small (about 200-800 Angstroms) unilamelar type in which the lipid content is greater than about 30 mol. % cholesterol, the selected proportion being adjusted for the optimal therapy.

An effective amount of a molecule of the present invention to be employed therapeutically will depend, for example, upon the therapeutic objectives, the route of administration, and the condition of the patient. Accordingly, it will be necessary for the therapist to titer the dosage and modify the route of administration as required to obtain the optimal therapeutic effect. A typical daily dosage might range from about 1 µg/kg to up to 100 mg/kg or more, depending on the factors mentioned above. Typically, the clinician will administer a molecule of the present invention until a dosage is reached that provides the required biological effect. The progress of this therapy is easily monitored by conventional assay techniques. If the purpose of the treatment is weight reduction, the therapy is normally continued until a desired body weight is reached.

Non-therapeutic uses of the OB protein-immunoglobulin fusions of the present invention include their use to identify and purify OB receptors. The identification and expression cloning of an OB receptor, using an OB protein-immunoadhesin is described in a Reference Example hereinbelow.

The invention will be further illustrated by the following non-limiting examples.

Example 1

Expression of OB- immunoadhesins

Using protein engineering techniques, the human OB protein was expressed as a fusion with the hinge, CH2 and CH3 domains of IgG-1. DNA constructs encoding the chimera of the human OB protein and IgG-1 Fc domains were made with the Fc region clones of human IgG-1. Human OB cDNA was obtained by PCR from human fat cell dscDNA (Clontech Buick-Clone cDNA product). The source of the IgG-1 cDNA was the plasmid pBSSK-CH_2CH_3. The chimera contained the coding sequence of the full length OB protein (amino acids 1-167 in Figure 5) and human IgG-1 sequences beginning at aspartic acid 216 (taking amino acid 114 as the first residue of the heavy chain constant region (Kabat *et al.*, Sequences of Proteins of Immunological

Interest 4th ed. [1987]), which is the first residue of the IgG-1 hinge after the cysteine residue involved in heavy-light chain bonding, and ending with residues 441 to include the CH2 and CH3 Fc domains of IgG-1. There was an insert of codons for three amino acids (GlyValThr) between the OB and IgG-1 coding sequences. If necessary, this short linker sequence can easily be deleted, for example by site directed deletion mutagenesis, to create an exact junction between the coding sequences of the OB protein and the IgG-1 hinge region. The coding sequence of the OB-IgG-1 immunoadhesin was subcloned into the pRK5-based vector pRK5tk-neo which contains a neomycine selectable marker, for transient expression in 293 cells using the calcium phosphate technique (Suva *et al.*, Science 237, 893-896 [1987]). 293 cells were cultured in HAM's : Low Glucose DMEM medium (50:50), containing 10% FBS and 2 mM L-Gln. For purification of OB-IgG-1 chimeras, cells were changed to serum free production medium PS24 the day after transfection and media collected after three days. The culture media was filtered.

The filtered 293 cell supernatant (400 ml) containing recombinant human OB-IgG-1 was made 1 mM in phenylmethylsulfonylfluoride and 2 µg/ml in aprotinin. This material was loaded at 4 °C onto a 1 x 4.5 cm Protein A agarose column (Pierce catalog # 20365) equilibrated in 100 mM HEPES pH 8. The flow rate was 75 ml/h. Once the sample was loaded, the column was washed with equilibration buffer until the A_{280} reached baseline. The OB-IgG-1 protein was eluted with 3.5 M $MgCl_2$ + 2% glycerol (unbuffered) at a flow rate of 15 ml/h. The eluate was collected with occasional mixing into 10 ml of 100 mM HEPES pH 8 to reduce the $MgCl_2$ concentration by approximately one-half and to raise the pH. The eluted protein was then dialyzed into phosphate buffered saline, concentrated, sterile filtered and stored either at 4°C or frozen at -70 °C. The OB-IgG-1 immunoadhesin prepared by this method is estimated by SDS-PAGE to be greater than 90% pure.

Example 2

Animal studies

A. Materials and Methods

OB protein Production -- Murine OB cDNA was obtained by PCR from an adipocyte cDNA library using primers based on the sequence of Zhang *et al.*, *supra*. Mature OB protein (amino acids 22-167) was expressed in E. coli by inserting the OB coding sequence in frame with the secretion sequence of the E. coli heat-stable enterotoxin II, downstream of the E. coli alkaline phosphatase promoter. Chang *et al.*, Gene 55, 189-96 (1987). After cell lysis, the insoluble fraction was solubilized in 8 M urea buffer pH 8.35 in the presence of 25 mM DTT. Reduced OB protein was purified by size exclusion and reverse phase HPLC, then refolded in the presence of glutathione. Refolded OB protein was purified by reverse phase HPLC and analyzed by SDS-PAGE and amino acid and mass spectrometry analyses.

Preparation of PEG-hOB -- The PEG derivatives of the human PB protein were prepared by reaction of hOB purified by reverse phase chromatography with a succinimidyl derivative of PEG propionic acid (SPA-PEG) having a nominal molecular weight of 10 kD, which had been obtained from Shearwater Polymers, Inc. (Huntsville, AL). After purification of the hOB protein by reverse phase chromatography, an approximately 1-2 mg/ml solution of the protein in 0.1% trifluoroacetic acid and approximately 40% acetonitrile, was diluted with 1/3 to 1/2 volume of 0.2 M borate buffer and the pH adjusted to 8.5 with NaOH. SPA-PEG was added to

the reaction mixture to make 1:1 and 1:2 molar ratios of protein to SPA-PEG and the mixture was allowed to incubate at room temperature for one hour. After reaction and purification by gel electrophoresis or ion exchange chromatography, the samples were extensively dialyzed against phosphate-buffered saline and sterilized by filtration through a 0.22 micron filter. Samples were stored at 4°C. Under these conditions, the PEG-hOB resulting from the 1:1 molar ratio protein to SPA-PEG reaction consisted primarily of molecules with one 10 kD PEG attached with minor amounts of the 2 PEG-containing species. The PEG-hOB from the 1:2 molar reaction consisted of approximately equal amounts of 2 and 3 PEGs attached to hOB, as determined by SDS gel electrophoresis. In both reactions, small amounts of unreacted protein was also detected. This unreacted protein can be efficiently removed by the gel filtration or ion exchange steps as needed. The PEG derivatives of the human OB protein can also be prepared essentially following the aldehyde chemistry described in EP 372,752 published June 13, 1990.

Animal Studies -- All manipulations involving animals were reviewed and approved by Genentech's Institutional Animal Care and Use Committee. Seven to eight week-old genetically obese C57Bl/6J-*ob/ob* (*ob/ob*) female mice were purchase from Jackson Labs (Bar Harbor, ME). Lean female mice of the same genetic background (C57Bl/6) were purchased from Harlan Sprague Dawley (Hollister, CA). Mice were housed in groups 3 - 6 with *ad libitum* access to water and standard mouse chow (Purina 5010; Purina Mills, Richmond, IN) in a temperature-, humidity- and light-controlled (lights on at 06:00h, of at 18:00h) colony room.

Miniosmotic pumps (Alzet model 2002; Alza Corp., Palo Alto, CA) were filled with purified recombinant OB protein (100 µg/kg/day) in sterile phosphate-buffered saline (PBS) or PBS alone under sterile conditions following manufacturer's instructions and incubated overnight in sterile saline at room temperature prior to implantation into mice. Mice were anesthetized with ketamine/xylazine, and miniosmotic pumps were implanted subcutaneously in the midscapular region. Daily subcutaneous injections of purified recombinant OB protein, hOB-IgG-1 fusion protein or PBS were made into the midscapular region of conscious mice. Injections were performed within one hour of lights out. The body weight of each mouse (to the nearest 0.1 gram) and the weight of the food contained in the food bin in each cage (to the nearest 0.1 gram) were recorded within one hour of lights out every one to two days. The data are depicted as the mean ± SEM. The number of animals is as described below and in the Figure legends.

B. Results with continuous subcutaneous infusion of OB protein

Lean female mice were treated with murine OB protein either as a continuous subcutaneous infusion or daily subcutaneous injections. The results are shown in Figure 1. The upper chart shows that the OB protein is significantly more effective in reducing body weight when delivered as a continuous infusion than when the same dose is delivered in the form of daily subcutaneous injections. The bottom chart shows the same difference in the ability of the OB protein to reduce adipose tissue weight.

C. Results with the OB-IgG-1 chimera

Obese female *ob/ob* mice were treated with human OB protein or with the human OB-IgG-1 chimera. The data are shown in Figure 2. The data presented in the top chart demonstrate that the hOB-IgG-1 fusion

protein is more potent than the native hOB protein at reducing body weight, when both proteins are administered similarly by daily subcutaneous infusion. It is noted that the increase in potency would be even more expressed, if the data were converted to molar amounts, as only about one third of the OB-IgG-1 chimera comes from the OB protein. The data further confirm the previous observation that continuous subcutaneous infusion (pump) or the hOB protein is more effective than daily subcutaneous injections (inj) at reducing body weight.

The data shown at the bottom chart of Figure 2 show that the hOB-IgG-1 fusion protein substantially reduced food intake. This result was unexpected as it was assumed that the fusion protein would be too large to cross the blood-brain barrier and exert its effect.

Obese (*ob/ob*) female mice were treated with either hOB or the hOB-IgG-1 chimera by daily subcutaneous injections for 7 days. The data shown in Figure 3 again demonstrate that the chimera is more effective than the native hOB protein at reducing body weight (top) and food intake (bottom).

In a further experiment, obese (*ob/ob*) female mice were treated with either the hOB-IgG-1 fusion protein, native hOB or hCD4-IgG-1 (control) by daily subcutaneous injections for seven days. The results shown in Figure 5 affirm that the hOB-IgG-1 fusion protein is more effective than the native hOB protein at reducing body weight (top and middle panels) and food intake (bottom panel).

D. Results with PEG-hOB

Obese female *ob/ob* mice were treated with human OB protein or with PEG derivatives of human OB. The data are shown in Figure 4. The data presented in the top chart demonstrate that PEG-hOB is more potent than the native hOB protein at reducing body weight, when both proteins are administered similarly by daily subcutaneous infusion.

The data shown at the bottom chart of Figure 4 show that the PEG-hOB proteins were substantially more effective in reducing food intake than unmodified native hOB.

Reference Example

Identification and cloning of an OB receptor

The OB protein-immunoadhesinof Example 1 was used to detect and expression clone an OB receptor. First, to identify a receptor source, several cell lines were screened with
1 µg/ml OB-IgG-1 fusion by flow cytometry. The detection system which consists of a biotin conjugated secondary antibody followed by streptavidin-phycoerythrinprovides a dramatic amplification of the signal and allows the detection of cells expressing low numbers of receptors. Two cell lines, human embryonic kidney 293 and human lung A549 cells were found to bind OB-IgG-1 but not an Flt-4 control immunoadhesin. Specific binding of OB-IgG-1 to the cells was also demonstrated by the addition of excess of bacterially expressed human OB protein. Addition of 10 µg/ml of human OB completely blocks the binding of OB-IgG-1 to 293 cells.

To isolate a cDNA encoding the OB receptor, COSN cells were transiently transfected with pools of approximately 10^5 clones of an oligo dT primed 293 cell cDNA library in pRK5B. Transfected cells were enriched by panning on plates coated with an anti-human Fc antibody after incubation with OB-IgG-1. After

three rounds of enrichment, 1 of 30 pools gave rise to OB-IgG-1 mediated adherence of COSN cells to the binding plates which could be competed by human leptin. cDNA clones picked randomly from this third round were transfected in pools of 10-20. Individual clones were finally identified after breaking down one pool of 10 that was scoring positive by panning.

Sequence analysis revealed a clone of approximately 5300 bp with an open reading frame encoding a protein of 896 amino acids. The sequence corresponded to a type 1 transmembrane protein with a 22 amino acid long signal peptide, 819 amino acid extracellular domain, 21 amino acid transmembrane domain and a short 34 amino acid intracellular domain. The sequence was found to essentially correspond to the human OB receptor identified and isolated by Tartaglia *et al.*, *supra*, and is identical with a human receptor sequence disclosed in copending application Serial No. 08/585,005 filed January 11, 1996.

While the invention has been illustrated by way of examples, the scope of the invention is not so limited. It will be understood that further modifications and variations are possible without diverting from the overall concept of the invention. All such modifications are intended to be within the scope of the present invention.

All references cited throughout the specification, including the examples, and the references cited therein are hereby expressly incorporated by reference.

SEQUENCE LISTING

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(ii) TITLE OF INVENTION: OB Protein Derivatives

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: 3.5 inch, 1.44 Mb floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: WinPatin (Genentech)

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7127 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double

(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TTCGAGCTCG CCCGACATTG ATTATTGACT AGTTATTAAT AGTAATCAAT 50

TACGGGGTCA TTAGTTCATA GCCCATATAT GGAGTTCCGC GTTACATAAC 100

TTACGGTAAA TGGCCCGCCT GGCTGACCGC CCAACGACCC CCGCCCATTG 150

ACGTCAATAA TGACGTATGT TCCCATAGTA ACGCCAATAG GGACTTTCCA 200

TTGACGTCAA TGGGTGGAGT ATTTACGGTA AACTGCCCAC TTGGCAGTAC 250

ATCAAGTGTA TCATATGCCA AGTACGCCCC CTATTGACGT CAATGACGGT 300

AAATGGCCCG CCTGGCATTA TGCCCAGTAC ATGACCTTAT GGGACTTTCC 350

TACTTGGCAG TACATCTACG TATTAGTCAT CGCTATTACC ATGGTGATGC 400

GGTTTTGGCA GTACATCAAT GGGCGTGGAT AGCGGTTTGA CTCACGGGGA 450

TTTCCAAGTC TCCACCCCAT TGACGTCAAT GGGAGTTTGT TTTGGCACCA 500

AAATCAACGG GACTTTCCAA AATGTCGTAA CAACTCCGCC CCATTGACGC 550

AAATGGGCGG TAGGCGTGTA CGGTGGGAGG TCTATATAAG CAGAGCTCGT 600

TTAGTGAACC GTCAGATCGC CTGGAGACGC CATCCACGCT GTTTTGACCT 650

CCATAGAAGA CACCGGGACC GATCCAGCCT CCGCGGCCGG GAACGGTGCA 700

TTGGAACGCG GATTCCCCGT GCCAAGAGTG ACGTAAGTAC CGCCTATAGA 750

GTCTATAGGC CCACCCCCTT GGCTTCGTTA GAACGCGGCT ACAATTAATA 800

CATAACCTTA TGTATCATAC ACATACGATT TAGGTGACAC TATAGAATAA 850

CATCCACTTT GCCTTTCTCT CCACAGGTGT CCACTCCCAG GTCCAACTGC 900

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CTTTGGCCCT ATCTTTTCTA TGTCCAAGCT GTGCCCATCC AAAAAGTCCA 1000

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GTGCCCAGCA CCTGAACTCC TGGGGGGACC GTCAGTCTTC CTCTTCCCCC 1500

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CAGGTCAGCC TGACCTGCCT GGTCAAAGGC TTCTATCCCA GCGACATCGC 1900

CGTGGAGTGG GAGAGCAATG GGCAGCCGGA GAACAACTAC AAGACCACGC 1950

CTCCCGTGCT GGACTCCGAC GGCTCCTTCT TCCTCTACAG CAAGCTCACC 2000

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CAGCACTGGG GCCAGATGGT AAGCCCTCCC GTATCGTAGT TATCTACACG 6100

ACGGGGAGTC AGGCAACTAT GGATGAACGA AATAGACAGA TCGCTGAGAT 6150

AGGTGCCTCA CTGATTAAGC ATTGGTAACT GTCAGACCAA GTTTACTCAT 6200

ATATACTTTA GATTGATTTA AAACTTCATT TTTAATTTAA AAGGATCTAG 6250

GTGAAGATCC TTTTTGATAA TCTCATGACC AAAATCCCTT AACGTGAGTT 6300

TTCGTTCCAC TGAGCGTCAG ACCCCGTAGA AAAGATCAAA GGATCTTCTT 6350

GAGATCCTTT TTTTCTGCGC GTAATCTGCT GCTTGCAAAC AAAAAAACCA 6400

CCGCTACCAG CGGTGGTTTG TTTGCCGGAT CAAGAGCTAC CAACTCTTTT 6450

TCCGAAGGTA ACTGGCTTCA GCAGAGCGCA GATACCAAAT ACTGTCCTTC 6500

TAGTGTAGCC GTAGTTAGGC CACCACTTCA AGAACTCTGT AGCACCGCCT 6550

ACATACCTCG CTCTGCTAAT CCTGTTACCA GTGGCTGCTG CCAGTGGCGA 6600

TAAGTCGTGT CTTACCGGGT TGGACTCAAG ACGATAGTTA CCGGATAAGG 6650

CGCAGCGGTC GGGCTGAACG GGGGGTTCGT GCACACAGCC CAGCTTGGAG 6700

CGAACGACCT ACACCGAACT GAGATACCTA CAGCGTGAGC ATTGAGAAAG 6750

CGCCACGCTT CCCGAAGGGA GAAAGGCGGA CAGGTATCCG GTAAGCGGCA 6800

GGGTCGGAAC AGGAGAGCGC ACGAGGGAGC TTCCAGGGGG AAACGCCTGG 6850

TATCTTTATA GTCCTGTCGG GTTTCGCCAC CTCTGACTTG AGCGTCGATT 6900

TTTGTGATGC TCGTCAGGGG GGCGGAGCCT ATGGAAAAAC GCCAGCTGGC 6950

ACGACAGGTT TCCCGACTGG AAAGCGGGCA GTGAGCGCAA CGCAATTAAT 7000

GTGAGTTACC TCACTCATTA GGCACCCCAG GCTTTACACT TTATGCTTCC 7050

GGCTCGTATG TTGTGTGGAA TTGTGAGCGG ATAACAATTT CACACAGGAA 7100

ACAGCTATGA CCATGATTAC GAATTAA 7127

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 397 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met His Trp Gly Thr Leu Cys Gly Phe Leu Trp Leu Trp Pro Tyr
1 5 10 15

Leu Phe Tyr Val Gln Ala Val Pro Ile Gln Lys Val Gln Asp Asp
20 25 30

Thr Lys Thr Leu Ile Lys Thr Ile Val Thr Arg Ile Asn Asp Ile
35 40 45

Ser His Thr Gln Ser Val Ser Ser Lys Gln Lys Val Thr Gly Leu
50 55 60

Asp Phe Ile Pro Gly Leu His Pro Ile Leu Thr Leu Ser Lys Met
65 70 75

Asp Gln Thr Leu Ala Val Tyr Gln Gln Ile Leu Thr Ser Met Pro
80 85 90

Ser Arg Asn Val Ile Gln Ile Ser Asn Asp Leu Glu Asn Leu Arg
95 100 105

Asp Leu Leu His Val Leu Ala Phe Ser Lys Ser Cys His Leu Pro
110 115 120

Trp Ala Ser Gly Leu Glu Thr Leu Asp Ser Leu Gly Gly Val Leu
125 130 135

Glu Ala Ser Gly Tyr Ser Thr Glu Val Val Ala Leu Ser Arg Leu
140 145 150

Gln Gly Ser Leu Gln Asp Met Leu Trp Gln Leu Asp Leu Ser Pro
155 160 165

Gly Cys Gly Val Thr Asp Lys Thr His Thr Cys Pro Pro Cys Pro
170 175 180

Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro

185 190 195

Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr
200 205 210

Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe
215 220 225

Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys
230 235 240

Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val
245 250 255

Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys
260 265 270

Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr
275 280 285

Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr
290 295 300

Leu Pro Pro Ser Arg Glu Glu Met Thr Lys Asn Gln Val Ser Leu
305 310 315

Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu
320 325 330

Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro
335 340 345

Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu
350 355 360

Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys
365 370 375

Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser
380 385 390

Leu Ser Leu Ser Pro Gly Lys
395 397

CLAIMS:

1. A long half-life derivative of an OB protein retaining a biological property of a native OB protein.

2. The long half-life derivative of claim 1 capable of reducing body weight and/or food intake in an individual treated.

3. The derivative of claim 1 which is a derivative of a native human OB protein.

4. The derivative of claim 1 which is an OB-immunoglobulin chimera.

5. The derivative of claim 1 which is a native OB protein or an OB-immunoglobulin chimera modified with a nonproteinaceous polymer.

6. The derivative of claim 4 wherein the nonproteinaceous polymer is polyethylene glycol (PEG).

7. A composition for the treatment of a condition associated with the abnormal expression or function of the OB gene, or for eliciting a biological response mediated by an OB receptor, comprising an effective amount of an OB derivative of claim 1.

8. The composition of claim 7 effective for weight and/or appetite reduction.

9. The composition of claim 7 effective in the reduction of elevated insulin levels.

10. A method for the treatment of a condition associated with the abnormal expression or function of the OB gene, or for eliciting a biological response mediated by an OB receptor, comprising administering to an individual to be treated a derivative of claim 1.

11. The method of claim 10 wherein the condition to be treated is selected from the group consisting of obesity, bulemia, and Type I or II diabetes.

12. A method for inducing weight loss or appetite loss is a subject, comprising administering to said subject an effective amount of a derivative of claim 1.

13. A chimeric polypeptide comprising an OB protein amino acid sequence capable of binding to a native OB receptor, linked to an immunoglobulin sequence.

14. The chimeric polypeptide of claim 13 wherein said immunoglobulin sequence is a constant domain sequence.

15. The chimeric polypeptide of claim 14 wherein said OB protein is human.

16. The chimeric polypeptide of claim 15 wherein two OB polypeptide-IgG heavy chain fusions are linked to each other by at least one disulfide bond to yield a homodimeric immunoglobulin-like structure.

17. The chimeric polypeptide of claim 16 wherein at least one of said OB polypeptide-IgG heavy chain fusions is associated with an immunoglobulin light chain.

18. An isolated nucleic acid sequence encoding an OB protein-immunoglobulin fusion.

19. A replicable expression vector comprising the nucleic acid of claim 18.

20. A host cell transformed wih the replicable expression vector of claim 19.

21. A process comprising culturing the host cells of claim 16 so as to express the nucleic acid encoding an OB protein-immunoglobulin fusion.

22. The process of claim 21 wherein said host cells are cotransformed with nucleic acid encoding at least two OB protein-immunoglobulin fusions.

23. The process of claim 22 wherein said cells are further transformed with nucleic acid encoding at least one immunoglobulin light chain.

24. A method of treating a condition associated with the abnormal expression or function of the OB gene or for eliciting a biological response mediated by an OB receptor comprising administering to a patient a therapeutically effective amount of the chimeric polypeptide of claim 13.

25. The method of claim 20 wherein said condition is selected from the group consisting of obesity, bulemia and type I or II diabetes.

26. A composition for the treatment of obesity comprising an effective amount of a chimeric polypeptide of claim 13 in association with a pharmaceutically acceptable carrier.

27. A method for inducing the growth of cells expressing an OB receptor comprising contacting said cells with the OB derivative of claim 1.

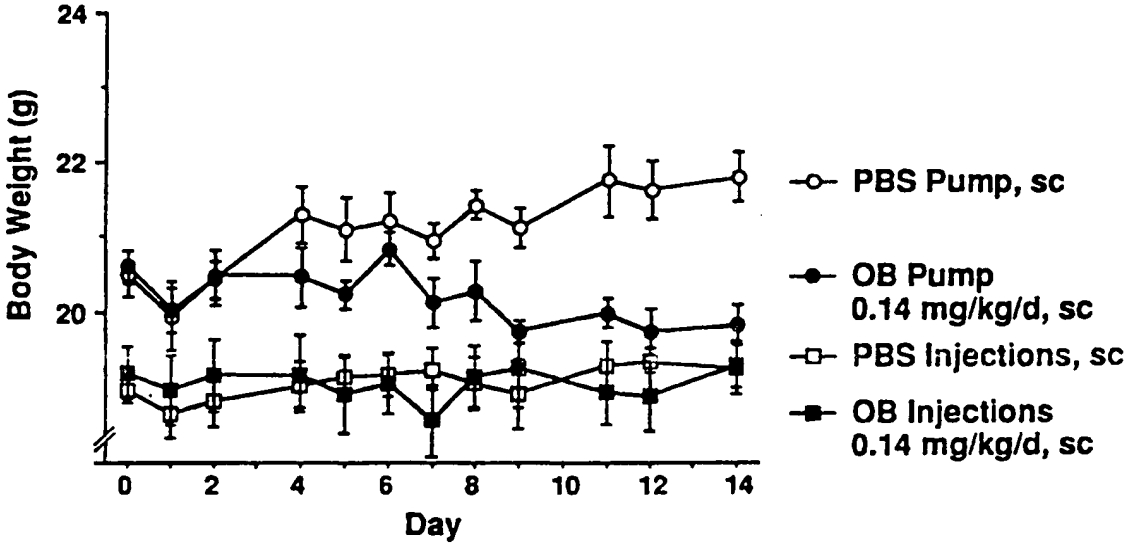

Body Weight (g)
24
22
20
0
2
4
6
8
10
12
14
Day
PBS Pump, sc
OB Pump
0.14 mg/kg/d, sc
PBS Injections, sc
OB Injections
0.14 mg/kg/d, sc

FIG. 1A

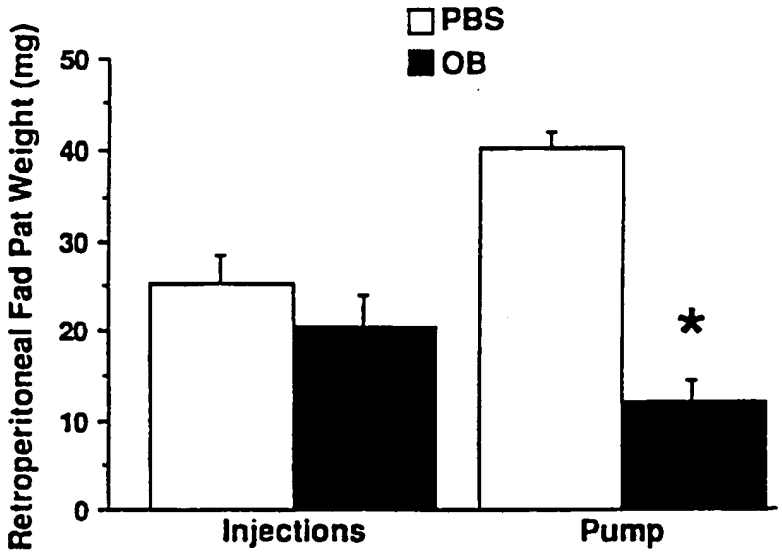

PBS
OB
Retroperitoneal Fad Pat Weight (mg)
50
40
30
20
10
0
*
Injections
Pump

FIG. 1B

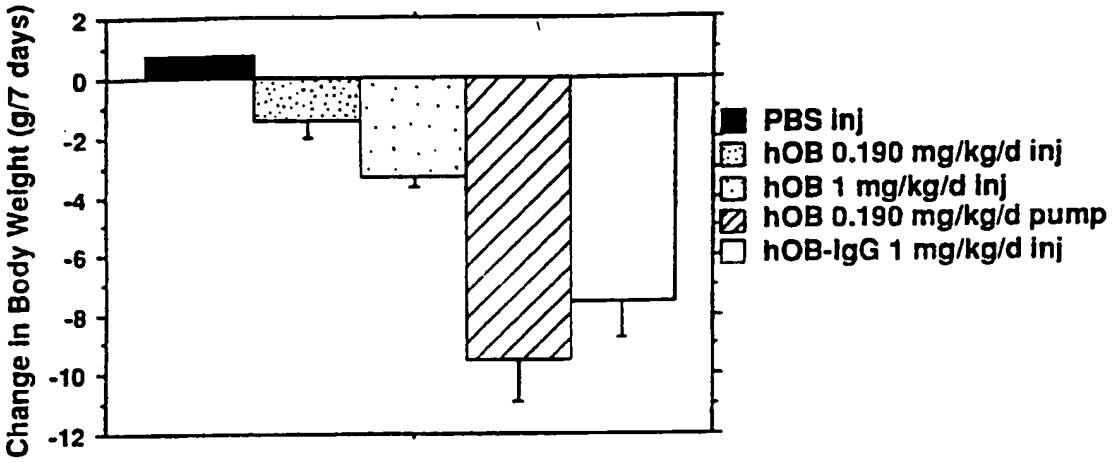

Change in Body Weight (g/7 days)
2
0
-2
-4
-6
-8
-10
-12
PBS inj
hOB 0.190 mg/kg/d inj
hOB 1 mg/kg/d inj
hOB 0.190 mg/kg/d pump
hOB-IgG 1 mg/kg/d inj

FIG. 2A

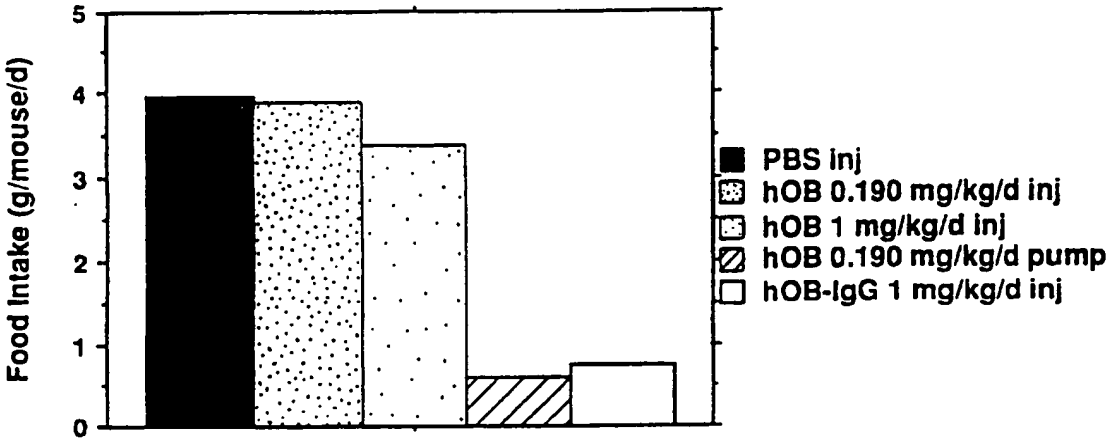

Food Intake (g/mouse/d)
5
4
3
2
1
0
PBS inj
hOB 0.190 mg/kg/d inj
hOB 1 mg/kg/d inj
hOB 0.190 mg/kg/d pump
hOB-IgG 1 mg/kg/d inj

FIG. 2B

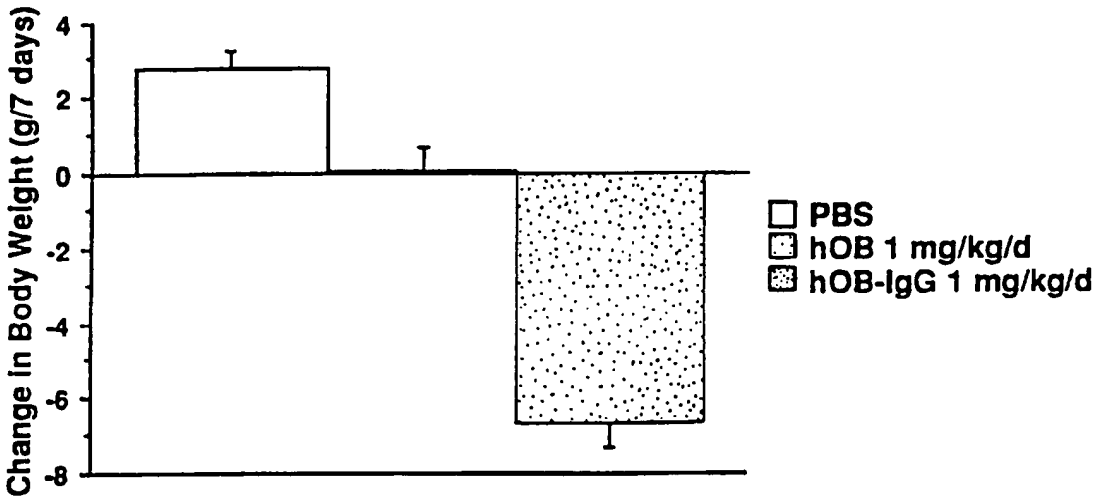

Change in Body Weight (g/7 days)
4
2
0
-2
-4
-6
-8
PBS
hOB 1 mg/kg/d
hOB-IgG 1 mg/kg/d

FIG. 3A

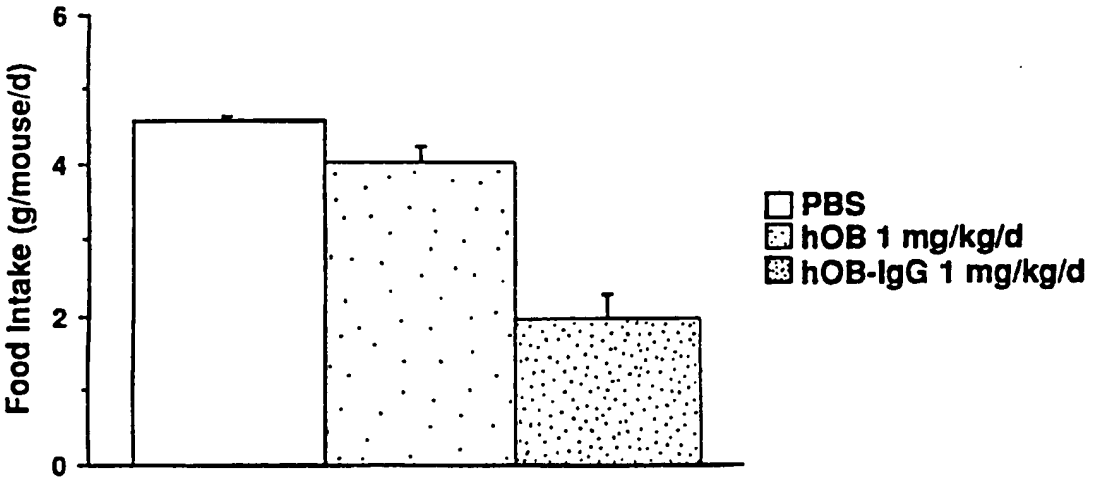

Food Intake (g/mouse/d)
6
4
2
0
PBS
hOB 1 mg/kg/d
hOB-IgG 1 mg/kg/d

FIG. 3B

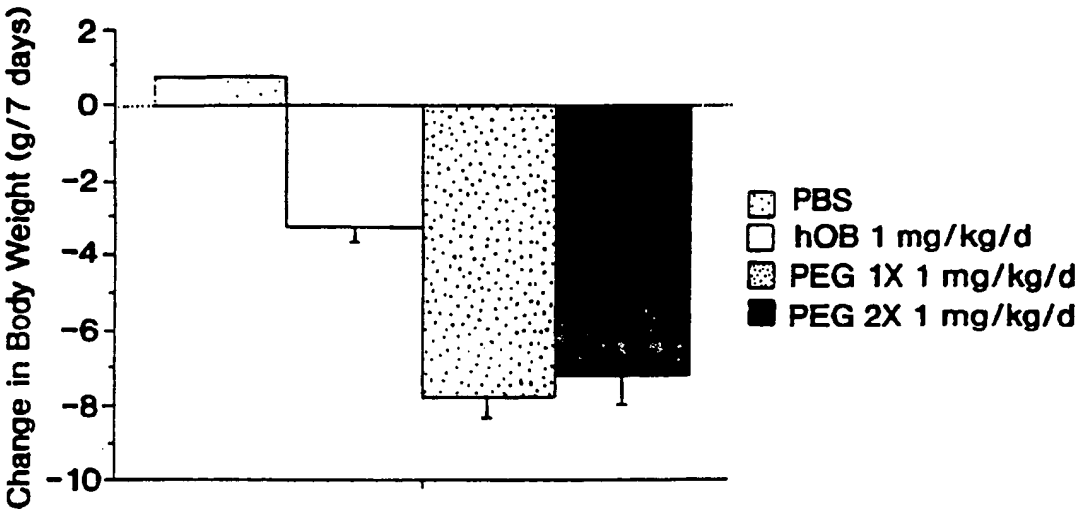

Change in Body Weight (g/7 days)
2
0
-2
-4
-6
-8
-10
PBS
hOB 1 mg/kg/d
PEG 1X 1 mg/kg/d
PEG 2X 1 mg/kg/d

FIG. 4A

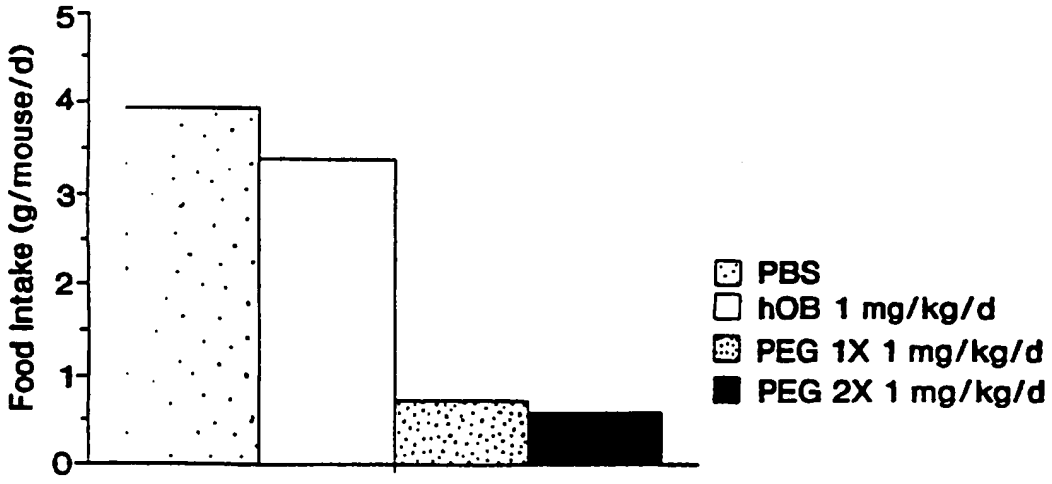

Food Intake (g/mouse/d)
5
4
3
2
1
0
PBS
hOB 1 mg/kg/d
PEG 1X 1 mg/kg/d
PEG 2X 1 mg/kg/d

FIG. 4B

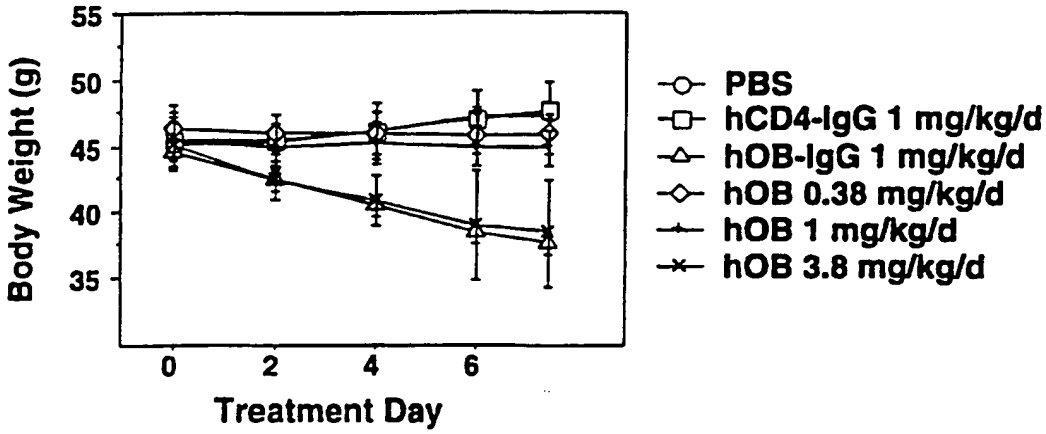


FIG. 5A

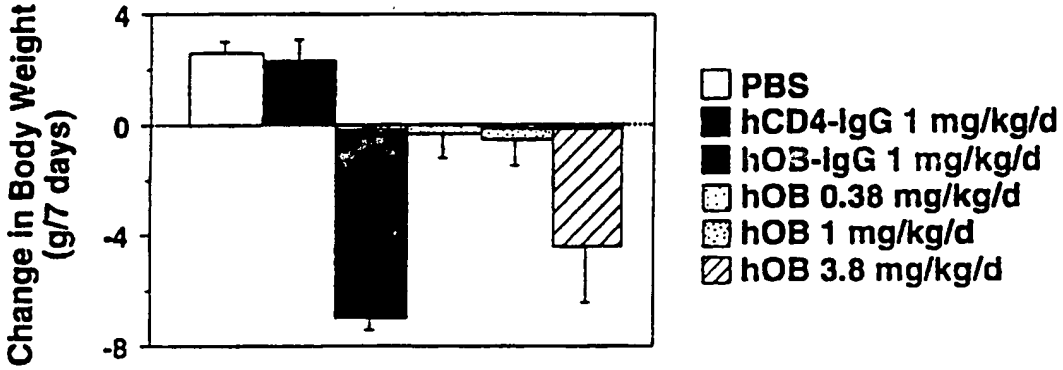


FIG. 5B

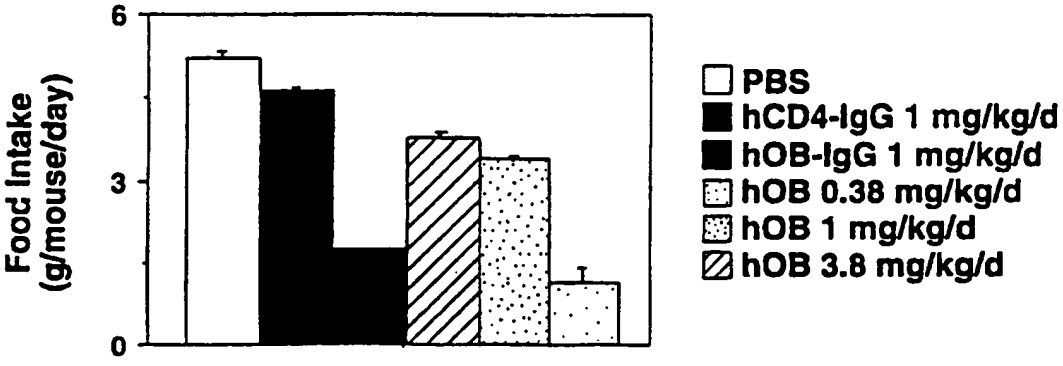


FIG. 5C

```
>                                     ss.pRK5tkneo.hOBIgC
> sites: std
> length: 7127 (circular)
>
>human OB ClaI/BstEII cloning
>CMV enhancer/promoter

        aluI
       sstI
       sacI
       hgiJII
       hgiAI/aspHI
       ecl136II
       bsp1286                                                                                     thaI
       bsiHKAI                                                                                     fnuDII/mvnI
       bmyI                      rmaI    tru9I                                                     bstUI
       banII                     maeI    mseI                                                      bsh1236I
     taqI                        speI    aseI/asnI/vspI                        bslI            aciI maeIII
  1 TTCGAGCTCG CCCGACATTG ATTATTGACT AGTTATTAAT AGTAATCAAT TACGGGGTCA TTAGTTCATA GCCCATATAT GGAGTTCCGC GTTACATAAC
    AAGCTCGAGC GGGCTGTAAC TAATAACTGA TCAATAATTA TCATTAGTTA ATGCCCCAGT AATCAAGTAT CGGGTATATA CCTCAAGGCG CAATGTATTG

                      scrFI
                      mvaI
                      ecoRII
                      dsaV
                   aciI
                 bglI bstNI                                 maeII
                sau96I                                   hinlI/acyI
                haeIII/palI     aciI                     ahaII/bsaHI
                asuI  apyI[dcm+]                aciI     aatII        maeII             maeIII
101 TTACGGTAAA TGGCCCGCCT GGCTGACCGC CCAACGACCC CCGCCCATTG ACGTCAATAA TGACGTATGT TCCCATAGTA ACGCCAATAG GGACTTTCCA
    AATGCCATTT ACCGGGCGGA CCGACTGGCG GGTTGCTGGG GGCGGGTAAC TGCAGTTATT ACTGCATACA AGGGTATCAT TGCGGTTATC CCTGAAAGGT

       maeII                                                                                      maeII
      hinlI/acyI                                                                                 hinlI/acyI
      ahaII/bsaHI                           rsaI                        rsaI           ahaII/bsaHI
      aatII                          bglI         csp6I            ndeI       csp6I          aatII
201 TTGACGTCAA TGGGTGGAGT ATTTACGGTA AACTGCCCAC TTGGCAGTAC ATCAAGTGTA TCATATGCCA AGTACGCCCC CTATTGACGT CAATGACGGT
    AACTGCAGTT ACCCACCTCA TAAATGCCAT TTGACGGGTG AACCGTCATG TAGTTCACAT AGTATACGGT TCATGCGGGG GATAACTGCA GTTACTGCCA
```

FIG. 6A

```
                   scrFI
                   mvaI
                   ecoRII
              aciI
            bglI  dsaV
           sau96I bstNI
           haeIII/palI           rsaI                                                    maeII                 styI        nlaIII
            asuI  apyI(dcm+)     csp6I                                          rsaI     snaBI                             ncoI
                              bsrI  nlaIII                                      csp6I    bsaAI                             dsaI hphI  aciI
                                                                                                                           bsaJI   sfaNI
301 AAATGGCCCG CCTGGCATTA TGCCCAGTAC ATGACCTTAT GGGACTTTCC TACTTGGCAG TACATCTACG TATTAGTCAT CGCTATTACC ATGGTGATGC
    TTTACCGGGC GGACCGTAAT ACGGGTCATG TACTGGAATA CCCTGAAAGG ATGAACCGTC ATGTAGATGC ATAATCAGTA GCGATAATGG TACCACTACG

                                                                                      maeII
                                                                                      hinII/acyI
                   rsaI                       pleI                                    ahaII/bsaHI            nlaIV
                   csp6I               aciI   hinfI                  bsmAI            aatII                  hgiCI
                                                                                                             banI
401 CGTTTTGGCA GTACATCAAT GGGCGTGGAT AGCGGTTTGA CTCACGGGGA TTTCCAAGTC TCCACCCCAT TGACGTCAAT GGGAGTTTGT TTTGGCACCA
    CCAAAACCGT CATGTAGTTA CCCGCACCTA TCGCCAAACT GAGTGCCCCT AAAGGTTCAG AGGTGGGGTA ACTGCAGTTA CCCTCAAACA AAACCGTGGT

                                                                                                             aluI
                                                                                                             sstI
                                                                                                             sacI
                                                                                                             hgiJII
                                                                                                             hgiAI/aspHI
                                                                                                             ecl136II
                                                                                                             bsp1286
                                                                                                             bsiHKAI
                                                                                  rsaI                       bmyI
                                 maeIII    aciI        hgaI         aciI          csp6I       mnlI           banII
501 AAATCAACGG GACTTTCCAA AATGTCGTAA CAACTCCGCC CCATTGACGC AAATGGGCGG TAGGCGTGTA CGGTGGGAGG TCTATATAAG CAGAGCTCGT
    TTTAGTTGCC CTGAAAGGTT TTACAGCATT GTTGAGGCGG GGTAACTGCG TTTACCCGCC ATCCGCACAT GCCACCCTCC AGATATATTC GTCTCGAGCA
```

FIG. 6B

```
                                                                                                          haeIII/palI
                                                                                                         mcrI
                                                                                                         eagI/xmaIII/eclXI
                                                                                                         eaeI
                                                                                                         cfrI
                                                                                                        fnu4HI
                                                                                                        aciI
                                                                                                       thaI
                        esp3I                                                                          fnuDII/mvnI
                   scrFI                                                   sau96I               sacII/sstII
                   mvaI bsmAI                                                    avaII                 nspBII
                   ecoRII                                                  asuI                 kspI   scrFI
                   dsaV                                                   nlaIV                 dsaI   nciI
                   bstNI  hinlI/acyI                                          scrFI              bglI bslI  mspI
                   apyI[dcm+]                                                 nciI           sau3AI mnlI bstUI
              sau3AI gsuI/bpmI                                                mspI           mboI/ndeII[dam-] hpaII
              mboI/ndeII[dam-]                                         mboII  hpaII          dpnI[dam+] bsaJI dsaV
              dpnI[dam+]  hgaI   fokI                                  bpuAI  dsaV           dpnII[dam-] bsh1236I
              dpnII[dam-] ahaII/bsaHI                       mnlI       bbsI   cauII          alwI[dam-] aciI  cauII
601 TTAGTGAACC GTCAGATCGC CTGGAGACGC CATCCACGCT GTTTTGACCT CCATAGAAGA CACCGGGACC GATCCAGCCT CCGCGGCCGG GAACGGTGCA
    AATCACTTGG CAGTCTAGCG GACCTCTGCG GTAGGTGCGA CAAAACTGGA GGTATCTTCT GTGGCCCTGG CTAGGTCGGA GGCGCCGGCC CTTGCCACGT
              ^Begin RNA

              tfiI
         aciI                                                                                       fnu4HI
        thaI hinfI                                                                                  aciI
        fnuDII/mvnI                                                          bstXI                 thaI
        bstUI                                        aciI                sau96I    styI            fnuDII/mvnI truGI
        bsh1236I                             maeII rsaI           pleI scfI haeIII/palI            bstUI        mseI
                                         maeIII    csp6I    scfI hinfI  asuI       bsaJI           bsh1236I   aseI/asnI/vspI
701 TTGGAACGCG GATTCCCCGT GCCAAGAGTG ACGTAAGTAC CGCCTATAGA GTCTATAGGC CCACCCCCTT GGCTTCGTTA GAACGCGGCT ACAATTAATA
    AACCTTGCGC CTAAGGGGCA CGGTTCTCAC TGCATTCATG GCGGATATCT CAGATATCCG GGTGGGGGAA CCGAAGCAAT CTTGCGCCGA TGTTAATTAT
                                                                                         ^sp6 promoter
```

FIG. 6C

```
                                                                                                              sau96I
                                                                                                              avaII
                                                                                                              asuI
                                                                                                           scrFI
                                                                                                           mvaI
                                                                                                           ecoRII
                                                                                                           dsaV
                                                                                                           bstNI
                                              maeIII                                                       apyI[dcm+]
                                             hphI    scfI        fokI                                 bslI bsaJI
801 CATAACCTTA TGTATCATAC ACATACGATT TAGGTGACAC TATAGAATAA CATCCACTTT GCCTTTCTCT CCACAGGTGT CCACTCCCAG GTCCAACTGC
    GTATTGGAAT ACATAGTATG TGTATGCTAA ATCCACTGTG ATATCTTATT GTAGGTGAAA CGGAAAGAGA GGTGTCCACA GGTGAGGGTC CAGGTTGACG
                                                          ^sp6 RNA start

                  ppu10I                         tfiI             sau96I
    mnlI        taqI nsiI/avaIII                 hinfI            haeIII/palI                     bsp1286
    bsaJI       claI/bsp106     nlaIV        aciI                 asuI                    aluI   bmyI fokI
901 ACCTCGGTTC TATCGATATG CATTGGGGAA CCCTGTGCGG ATTCTTGTGG CTTTGGCCCT ATCTTTTCTA TGTCCAAGCT GTGCCCATCC AAAAAGTCCA
    TGGAGCCAAG ATAGCTATAC GTAACCCCTT GGGACACGCC TAAGAACACC GAAACCGGGA TAGAAAAGAT ACAGGTTCGA CACGGGTAGG TTTTTCAGGT
  1                       Met HisTrpGlyT hrLeuCysGl yPheLeuTrp LeuTrpProT yrLeuPheTy rValGlnAla ValProIleG lnLysValGln
    ^cloning linker    ^human OB start

                                                 sau3AI
                                                 mboI/ndeII[dam-]
                                                 dpnI[dam+]
                                             scrFI
                                             mvaI
                                             ecoRII                                                          mspI
                                             dsaV                                                        hpaII
                                             bstNI                                                      cfr10I
                                             apyI[dcm+]                                                     bsaWI
                                                                                                            ageI
                                          hphI  dpnII[dam-]                                  mnlI          hphI
                  mnlI          munI    maeIII alwI[dam-]                             bsmAI               maeIII
1001 AGATGACACC AAAACCCTCA TCAAGACAAT TGTCACCAGG ATCAATGACA TTTCACACAC GCAGTCAGTC TCCTCCAAAC AGAAAGTCAC CGGTTTGGAC
     TCTACTGTGG TTTTGGGAGT AGTTCTGTTA ACAGTGGTCC TAGTTACTGT AAAGTGTGTG CGTCAGTCAG AGGAGGTTTG TCTTTCAGTG GCCAAACCTG
  29 AspAspThr LysThrLeuI leLysThrIl eValThrArg IleAsnAspI leSerHisTh rGlnSerVal SerSerLysG lnLysValTh rGlyLeuAsp
```

FIG. 6D

hgiJII
bsp1286
bmyI
banII
scrFI
mvaI alaIV
ecoRII
dsaV
bstNI
bsaJI
apyI[dcm+]
1101 TTCATTCCTG GGCTCCACCC CATCCTGACC TTATCCAAGA TGGACCAGAC ACTGGCAGTC TACCAACAGA TCCTCACCAG TATGCCTTCC AGAAACGTGA
AAGTAAGGAC CCGAGGTGGG GTAGGACTGG AATAGGTTCT ACCTCGTCTG TGACCGTCAG ATGGTTGTCT AGGAGTGGTC ATACGGAAGG TCTTTGCACT
62 PheIleProG lyLeuHisPr oIleLeuThr LeuSerLysH elAspGlnTh rLeuAlaVal TyrGlnGln leLeuThrSe rMetProSer ArgAsnValIle
hphI
mnlI
sau3AI bsrI
alwNI
pflMI
bslI
sau96I
avaII
asuI
bstYI/xhoII
haeIII/paII
sau96I styI
asuI gsuI/bpmI
scrFI scrFI
mvaI mvaI bsmAI
ecoRII ecoRII
dsaV dsaV
bstNI bstNI bsaJI
bsaJI bstXI apyI[dcm+]
apyI[dcm+] haeI bsaI
baeJI bsrI haeIII/paII
sau3AI pmlI
eco72I
bstYI/xhoII
bsaAI
gsuI/bpmI scrFI
scrFI nciI mboI/ndeII[dam-]
mvaI mspI dpnI[dam+]
ecoRII hpaII mboII maeII
dsaV dsaV dpnII[dam-]
bstNI mnlI alwI[dam-] bbrPI haeIII/paII bbvI
apyI[dcm+] cauII mboII[dam-] haeI ddeI aluI
bstXI
1201 TCCAAATATC CAGGACCTTG GAGAACCTCC GGGATCTTCT TCACGTGCTG GCCTTCTCTA AGACCTGCCA CTTGCCCCTG GCCAGTGGCC TGGAGACCTT
AGGTTTATAG GTCCTGGAAC CTCTTGGAGG CCCTAGAAGA AGTGCACGAC CGGAAGAGAT TCTCGACGGT GAACGGGACC CGGTCACCGG ACCTCTGGAA
96 GlnIleSe rAsnAspLeu GluAsnLeuA rgAspLeuLe uHisValLeu AlaPheSerL ysSerCysHi sLeuProTrp AlaSerGlyL euGluThrLeu
ddeI scfI
bslI pstI bsgI scfI
sau96I haeIII/paII fnu4HI bbvI
xcmI bstXI mnlI asuI
eco57I
scrFI
mvaI scrFI
ecoRII mvaI aluI
dsaV ecoRII
bstNI dsaV
bsaJI bstNI hindIII
apyI[dcm+] apyI[dcm+]
1301 GGACAGCCTG GGGGGTGTCC TGGAAGCTTC AGGCTACTCC ACAGAGGTGC TGCCCCTGAG CAGGCTGCAG GGGTCTCTGC AGGACATGCT GTGGCAGCTG
CCTGTCGGAC CCCCCACACG ACCTTCGAAG TCCGATGAGG TGTCTCCACC ACCGGGACTC GTCCGACGTC CCCAGAGACG TCCTGTACGA CACCGTCGAC
129 AspSerLeu GlyGlyValL euGluAlaSe rGlyTyrSer ThrGluValV alAlaLeuSe rArgLeuGln GlySerLeuG lnAspMetLe uTrpGlnLeu
pvuII
nlaIII
nspI
nspBII
fnu4HI
bsmAI
bsaI bsgI
nspHI
bbvI asuI
sau96I
aviII
aluI

FIG. 6E

```
             scrFI                                                                      eam1105I
             mvaI                                                                            sau96I
             ecoRII                                                                scrFI
             dsaV                                                                  mvaI    avaII
             bstNI                                                                       ecoRII
             bslI                                                                        dsaV
             bsaJI          hphI                nlaIII                                   bstNI    asuI       mboII  mboII
       ddeI  apyI(dcm+)    maeIII             nspI           bsp1286                     bsaJI  nlaIV       bpuAI  earI/ksp632I
      mnlI  bsaJI     aclI bstEII             nspHI          bmyI alwNI                  apyI(dcm+)         bbsI mnlI
1401 GACCTCAGCC CTGGGTGCGG GGTCACCGAC AAAACTCACA CATGCCCACC GTGCCCAGCA CCTGAACTCC TGGGGGGACC GTCAGTCTTC CTCTTCCCCC
     CTGGAGTCGG GACCCACGCC CCAGTGGCTG TTTTGAGTGT GTACGGGTGG CACGGGTCGT GGACTTGAGG ACCCCCCTGG CAGTCAGAAG GAGAAGGGGG
 162 AspLeuSerP roGlyCysGl yValThrAsp LysThrHisT hrCysProPr oCysProAla ProGluLeuL euGlyGlyPr oSerValPhe LeuPheProPro
                       ^insertion of a gly
                               ^START OF HUMAN IgG1 CH2CH3

                                 sau96I
                                 nlaIV
                              mspI
                              hpaII
                             scrFI
                             nciI
                             dsaV
                        sau3AI   avaII
                        mboI/ndeII(dam-)          nlaIII
                     nlaIII   cauII      mnlI    nspI                               drdI   mnlI               maeII
                    rcaI dpnI(dam+)     ddeI     nspHI                            mboII  ddeI                rsaI
        styI        bspHI(dam-)    asuI eco81I maeIII                              bpuAI  eco81I              csp6I
        bsaJI     mnlI   dpnII(dam-)   bsu36I/mstII/sauI          maeII            bbsI  bsu36I/mstII/sauI bsrI bsaAI
1501 CAAAACCCAA GGACACCCTC ATGATCTCCC GGACCCCTGA GGTCACATGC GTGGTGGTGG ACGTGAGCCA CGAAGACCCT GAGGTCAAGT TCAACTGGTA
     GTTTTGGGTT CCTGTGGGAG TACTAGAGGG CCTGGGGACT CCAGTGTACG CACCACCACC TGCACTCGGT GCTTCTGGGA CTCCAGTTCA AGTTGACCAT
 196   LysProLy sAspThrLeu MetIleSerA rgThrProGl uValThrCys ValValValA spValSerHi sGluAspPro GluValLysP heAsnTrpTyr
```

FIG. 6F

1601 CGTGGACGGGC GTGGAGGTGC ATAATGCCAA GACAAAGCCG CGGGACGAGC AGTACAACAG CACGTACCGT GTGGTCAGCG TCCTCACCGT CCTGCACCAG
GCACCTGCCG CACCTCCACG TATTACGGTT CTGTTTCGGC GCCCTCCTCG TCATGTTGTC GTGCATGGCA CACCAGTCGC AGGAGTGGCA GGACGTGGTC
229 ValAspGly ValGluValH isAsnAlaLy sThrLysPro ArgGluGluG lnTyrAsnSe rThrTyrArg ValValSerV alLeuThrVa lLeuHisGln

1701 GACTGGCTGA ATGGCAAGGA GTACAAGTGC AAGGTCTCCA ACAAAGCCCT CCCAGCCCCC ATCGAGAAAA CCATCTCCAA AGCCAAAGGG CAGCCCCGAG
CTGACCGACT TACCGTTCCT CATGTTCACG TTCCAGAGGT TGTTTCGGGA GGGTCGGGGG TAGCTCTTTT GGTAGAGGTT TCGGTTTCCC GTCGGGGCTC
262 AspTrpLeuA snGlyLysGl uTyrLysCys LysValSerA snLysAlaLe uProAlaPro IleGluLysT hrIleSerLy sAlaLysGly GlnProArgGlu

1801 AACCACAGGT GTACACCCTG CCCCCATCCC GGGAAGAGAT GACCAAGAAC CAGGTCAGCC TGACCTGCCT GGTCAAAGGC TTCTATCCCA GCGACATCGC
TTGGTGTCCA CATGTGGGAC GGGGGTAGGG CCCTTCTCTA CTGGTTCTTG GTCCAGTCGG ACTGGACGGA CCAGTTTCCG AAGATAGGGT CGCTGTAGCG
296 ProGlnVa lTyrThrLeu ProProSerA rgGluGluMe tThrLysAsn GlnValSerL euThrCysLe uValLysGly PheTyrProS erAspIleAla

FIG. 6G

```
                                   mspI
                                   hpaII                                                                                              dsaI
                                fnu4HI                                              pleI                    mnlI                   bphI
                                bbvI                               mnlI             hinfI      nlaIV  mboII     scfI        aluI   bsaJI
1901 CGTGGAGTGG GAGAGCAATG GGCAGCCGGA GAACAACTAC AAGACCACGC CTCCCGTGCT GGACTCCGAC GGCTCCTTCT TCCTCTACAG CAAGCTCACC
     GCACCTCACC CTCTCGTTAC CCGTCGGCCT CTTGTTGATG TTCTGGTGCG GAGGGCACGA CCTGAGGCTG CCGAGGAAGA AGGAGATGTC GTTCGAGTGG
 329  ValGluTrp GluSerAsnG lyGlnProGl uAsnAsnTyr LysThrThrP roProValLe uAspSerAsp GlySerPheP heLeuTyrSe rLysLeuThr

                                                                                                                                  scrFI
                                                                                                                                  ncil
                                           mboII                                                                                  mspI
                                          bpuAI                          nlaIII                                                   hpaII
                                      maeII                         ppu10I                                    sapI                dsaV
                        fnu4HI     xmnI bbsI                        nsiI/avaIII                               mboII mnlI      bsmAI
                  bspHI bbvI      asp700       nlaIII         sfaNI    mnlI                                   earI/ksp632I bslI  cauII
2001 GTGGACAAGA GCAGGTGGCA GCAGGGGAAC GTCTTCTCAT GCTCCGTGAT GCATGAGGCT CTGCACAACC ACTACACGCA GAAGAGCCTC TCCCTGTCTC
     CACCTGTTCT CGTCCACCGT CGTCCCCTTG CAGAAGAGTA CGAGGCACTA CGTACTCCGA GACGTGTTGG TGATGTGCGT CTTCTCGGAG AGGGACAGAG
 362 ValAspLysS erArgTrpGl nGlnGlyAsn ValPheSerC ysSerValMe tHisGluAla LeuHisAsnH isTyrThrGl nLysSerLeu SerLeuSerPro

                                                                                                     sau96I
                                                                                                   nlaIII
                                       taqI                                                  fnu4HI   haeIII/palI
                                      salI                               taqI                bglI styI
                                    pleI     scfI                     pleI      scfI          sfiI  ncoI
                                 rmaI hincII/hindII                rmaI  salI  pstI          eaeI   dsaI
                            sau96I  hinfI    pstI               xbaI   hincII/hindII          cfrI   bsaJI                  aluI
                            haeIII/palI      bsgI         aluI maeI   accI  bsgI       aluI haeIII/palI                   fnu4HI
                            asuI maeI accI bspHI     hindIII     hinfI  bspHI        hindIII  aciI   asuI                 bbvI
2101 CGGGTAAATG AGTGCGACGG CCCTAGAGTC GACCTGCAGA AGCTTCTAGA GTCGACCTGC AGAAGCTTGG CCGCCATGGC CCAACTTGTT TATTGCAGCT
     GCCCATTTAC TCACGCTGCC GGGATCTCAG CTGGACGTCT TCGAAGATCT CAGCTGGACG TCTTCGAACC GGCGGTACCG GGTTGAACAA ATAACGTCGA
 396   GlyLys
                                                                                                      ^sv40 early poly A

                                                                                  rmaI
             maeIII               sfaNI     apoI                                bsmI maeI
2201 TATAATGGTT ACAAATAAAG CAATAGCATC ACAAATTTCA CAAATAAAGC ATTTTTTTCA CTGCATTCTA GTTGTGGTTT GTCCAAACTC ATCAATGTAT
     ATATTACCAA TGTTTATTTC GTTATCGTAG TGTTTAAAGT GTTTATTTCG TAAAAAAAGT GACGTAAGAT CAACACCAAA CAGGTTTGAG TAGTTACATA
```

FIG. 6H

```
                   sau3AI
                   mboI/ndeII[dam-]
                   dpnI[dam+]
                   dpnII[dam-]
                  pvuI/bspCI
                  mcrI                                                                                   rsaI
                 taqI[dam-]  tru9I                haeIII/palI                                            csp6I
                claI/bsp106[dam-]                haeI                                                   nlaIV
               sau3AI      mseI       fnu4HI styI                                                       kpnI
               mboI/ndeII[dam-]        bbvI   ncoI                                                      hgiCI
               dpnI[dam+] xmnI        hinPI   dsaI                                                      banI
               dpnII[dam-]  aseI/asnI/vspI    bsaJI                                                     asp718     mnlI
      nlaIII  alwI[dam-]  asp700      hhaI/cfoI nlaIII          mnlI       mnlI                         acc65I  ddeI  aciI
2301 CTTATCATGT CTGGATCGAT CGGGAATTAA TTCGGCGCAG CACCATGGCC TGAAATAACC TCTGAAAGAG GAACTTGGTT AGGTACCTTC TGAGGCGGAA
     GAATAGTACA GACCTAGCTA GCCCTTAATT AAGCCGCGTC GTGGTACCGG ACTTTATTGG AGACTTTCTC CTTGAACCAA TCCATGGAAG ACTCCGCCTT
                            ^sv40 origin

                                                 nlaIV
                                               scrFI                          sfaNI                 scrFI
                                               mvaI                              ppu10I                  mvaI
                                               ecoRII                       nsiI/avaIII             ecoRII
                                               dsaV                        nlaIII                   dsaV
       aluI                                    bstNI                          sphI                       bstNI
      pvuII                                    apyI[dcm+]                     nspI                       apyI[dcm+]
      nspBII                                  bsaJI                           nspHI                     sexAI
2401 AGAACCAGCT GTGGAATGTG TGTCAGTTAG GGTGTGGAAA GTCCCCAGGC TCCCCAGCAG GCAGAAGTAT GCAAAGCATG CATCTCAATT AGTCAGCAAC
     TCTTGGTCGA CACCTTACAC ACAGTCAATC CCACACCTTT CAGGGGTCCG AGGGGTCGTC CGTCTTCATA CGTTTCGTAC GTAGAGTTAA TCAGTCGTTG

                     nlaIV
                   scrFI
                   mvaI                             ppu10I
                   ecoRII                        nsiI/avaIII
                   dsaV                         nlaIII
                   bstNI                           sphI
                   apyI[dcm+]                      nspI sfaNI
                  bsaJI                            nspHI                            aciI         aciI fokI
2501 CAGGTGTGGA AAGTCCCCAG GCTCCCCAGC AGGCAGAAGT ATGCAAAGCA TGCATCTCAA TTAGTCAGCA ACCATAGTCC CGCCCCTAAC TCCGCCCATC
     GTCCACACCT TTCAGGGGTC CGAGGGGTCG TCCGTCTTCA TACGTTTCGT ACGTAGAGTT AATCAGTCGT TGGTATCAGG GCGGGGATTG AGGCGGGTAG
```

FIG. 6I

```
                                                                                          fnu4HI
                                                                                          bglI
                                                                                         sfiI
                                                  nlaIII                                 haeIII/palI
                                                 styI                                  mnlI     mnlI     ddeI
                       bsrI                      ncoI                                haeIII/palI bsaJI mnlI   aluI
      aciI        aciI           aciI       bslI dsaI                              mnlI bsaJI aciI    haeIII/palI
                                           aciI bsaJI
2601 CCGCCCCTAA CTCCGCCCAG TTCCGCCCAT TCTCCGCCCC ATGGCTGACT AATTTTTTTT ATTTATGCAG AGGCCGAGGC CGCCTCGGCC TCTGAGCTAT
     GGCGGGGATT GAGGCGGGTC AAGGCGGGTA AGAGGCGGGG TACCGACTGA TTAAAAAAAA TAAATACGTC TCCGGCTCCG GCGGAGCCGG AGACTCGATA

                                                                                                  scrFI
                                                                                                  mvaI
                                                                                              sau96I
                                                                                              nlaIV
                                                                                              avaII   sau96I
                                                                                            thaI    ecoRII
                                          styI                                              fnuDII/mvnI
                                          bsaJI                     sfuI                    bstUI   dsaV
                                          blnI                      bstBI                   bsh1236I  avaII
                                        haeIII/palI                 bsiCI                  hinPI    bstNI
                                       stuI rmaI                    asuII                  hhaI/cfoI  asuI
                  mnlI                 haeI maeI                tru9I                    fnu4HI asuI apyI[dcm+]
                mnlI                   mnlI avrII         aluI  mseI taqI       sfaNI      aciI aciI  bsaJI
2701 TCCAGAAGTA GTGAGGAGGC TTTTTTGGAG GCCTAGGCTT TTGCAAAAAG CTGTTAATTC GAACACGCAG ATGCAGTCGG GGCGGCGCGG TCCCAGGTCC
     AGGTCTTCAT CACTCCTCCG AAAAAACCTC CGGATCCGAA AACGTTTTTC GACAATTAAG CTTGTGCGTC TACGTCAGCC CCGCCGCGCC AGGGTCCAGG
                                                       ^start pUC118
                                                               ^TK promoter

                                                                                                   sau3AI
                                                                                                   mboI/ndeII[dam-]
                                                                                                   dpnI[dam+]
                       thaI                                                                    sau3AI
                       fnuDII/mvnI                                                             mboI/ndeII[dam-]
                       bstUI                                                                   dpnI[dam+]
                      mluI                              fnu4HI                                 bstYI/xhoII
                      aflIII                            bbvI                                   bglII  dpnII[dam-]
                   hphI bsh1236I  mnlI                 scfI                                         
                tru9I  hgaI     haeIII/palI            pstI            tru9I  hincII/hindII aciI dpnII[dam-] bsmAI
                mseI maeIII    haeI  taqI              bsgI       aciI mseI  hgaI       fnu4HI    bclI[dam-]
2801 ACTTCGCATA TTAAGGTGAC GCGTGTGGCC TCGAACACCG AGCGACCCTG CAGCGACCCG CTTAACAGCG TCAACAGCGT GCCGCAGATC TGATCAAGAG
     TGAAGCGTAT AATTCCACTG CGCACACCGG AGCTTGTGGC TCGCTGGGAC GTCGCTGGGC GAATTGTCGC AGTTGTCGCA CGGCGTCTAG ACTAGTTCTC
                                                                  tn5 neomycin phosphotransferase gene.  ^
```

FIG. 6J

```
                                                                            aciI
           sau3AI                                                           fnu4HI
           mboI/ndeII[dam-]                                                 haeIII/palI
           dpnI[dam+]                                                       mcrI
           dpnII[dam-]                                                      eagI/xmaIII/eclXI
       mnlI                                                                 eaeI
     mamI[dam-]                                                             cfrI                                            bsp1286
     bsaBI[dam-]                                                            mspI                                            bmyI
    fokI  alwI[dam-]   nlaIII                                  bspHI        hpaII                mnlI                    bsrI
2901 ACAGGATGAG GATCGTTTCG CATGATTGAA CAAGATGGAT TGCACGCAGG TTCTCCGGCC GCTTGGGTGG AGAGGCTATT CGGCTATGAC TGGGCACAAC
     TGTCCTACTC CTAGCAAAGC GTACTAACTT GTTCTACCTA ACGTGCGTCC AAGAGGCCGG CGAACCCACC TCTCCGATAA GCCGATACTG ACCCGTGTTG

                                                               hinPI
                                                               hhaI/cfoI
                                                               nlaIV
                                                               narI scrFI
                                                               kasI nciI                                 bsp1286
                                                               hinlI/acyI                                bmyI
                                                               hgiCI mspI                                nlaIV
                                                               haeII hpaII                               hgiCI
                                                               banI dsaV                                 banI
                         aciI                                  banI dsaV                               mspI            scfI
         fnu4HI          fnu4HI         mspI        hinPI      ahaII/bsaHI                             hpaII           pstI
         bbvI      sfaNI    bslI        hpaII       hhaI/cfoI   cauII                 drdI            bsaWI           bsgI
3001 AGACAATCGG CTGCTCTGAT GCCGCCGTGT TCCGGCTGTC AGCGCAGGGG CGCCCGGTTC TTTTTGTCAA GACCGACCTG TCCGGTGCCC TGAATGAACT
     TCTGTTAGCC GACGAGACTA CGGCGGCACA AGGCCGACAG TCGCGTCCCC GCGGGCCAAG AAAAACAGTT CTGGCTGGAC AGGCCACGGG ACTTACTTGA

                                                                            hgiAI/aspHI
                                                                            bsp1286
               fnu4HI                                                   aluI
              thaI                                                     pvuII
              fnuDII/mvnI                                             fnu4HI
              bstUI                                                   bbvI  bsiHKAI
              bsh1236I                haeIII/palI                    hinPI  bmyI
             hinPI                   mscI/balI                       hhaI/cfoI        maeII      eco57I
          fnu4HI                     haeI                          mstI nspBII       tth111I/aspI                     fnu4HI
          bbvI aciI                  eaeI                          aviII/fspI       taqI      maeIII    aciI      bsrI bbvI
      mnlI  hhaI/cfoI               cfrI
3101 GCAGGACGAG GCAGCGCGGC TATCGTGGCT GGCCACGACG GGCGTTCCTT GCGCAGCTGT GCTCGACGTT GTCACTGAAG CGGGAAGGGA CTGGCTGCTA
     CGTCCTGCTC CGTCGCGCCG ATAGCACCGA CCGGTGCTGC CCGCAAGGAA CGCGTCGACA CGAGCTGCAA CAGTGACTTC GCCCTTCCCT GACCGACGAT
```

FIG. 6K

3201 TTGGGCGAAG TGCCCGGGCA GCATCTCCTG TCATCTCACC TTGCTCCTGC CGAGAAAGTA TCCATCATGG CTGATGCAAT GCGCGGGCTG CATACGCTTG
AACCCGCTTC ACGGGCCCGT CGTAGAGGAC AGTAGAGTGG AACGAGGACG GCTCTTTCAT AGGTAGTACC GACTACGTTA CGCCGCCGAC GTATGCGAAC
3301 ATCCGGCTAC CTGCCCATTC GACCACCAAG CGAAACATCG CATCGAGCGA GCACGTACTC GGATGGAAGC CGGTCTTGTC GATCAGGATG ATCTGGACGA
TAGGCCGATG GACGGGTAAG CTGGTGGTTC GCTTTGTAGC GTAGCTCGCT CGTGCATGAG CCTACCTTCG GCCAGAACAG CTAGTCCTAC TAGACCTGCT
3401 AGAGCATCAG GGGCTCCGCC CAGCCGAACT GTTCGCCAGG CTCAAGCGCC GCATGCCCGA CGCCGAGGAT CTCGTCGTGA CCCATGGCGA TGCCTGCTTG
TCTCGTAGTC CCCGAGGCGG GTCGGCTTGA CAAGCGGTCC CAGTTCGCGG CGTACGGGCT GCGGCTCCTA GACCAGCACT GGGTACCGCT ACGGACGAAC

FIG. 6L

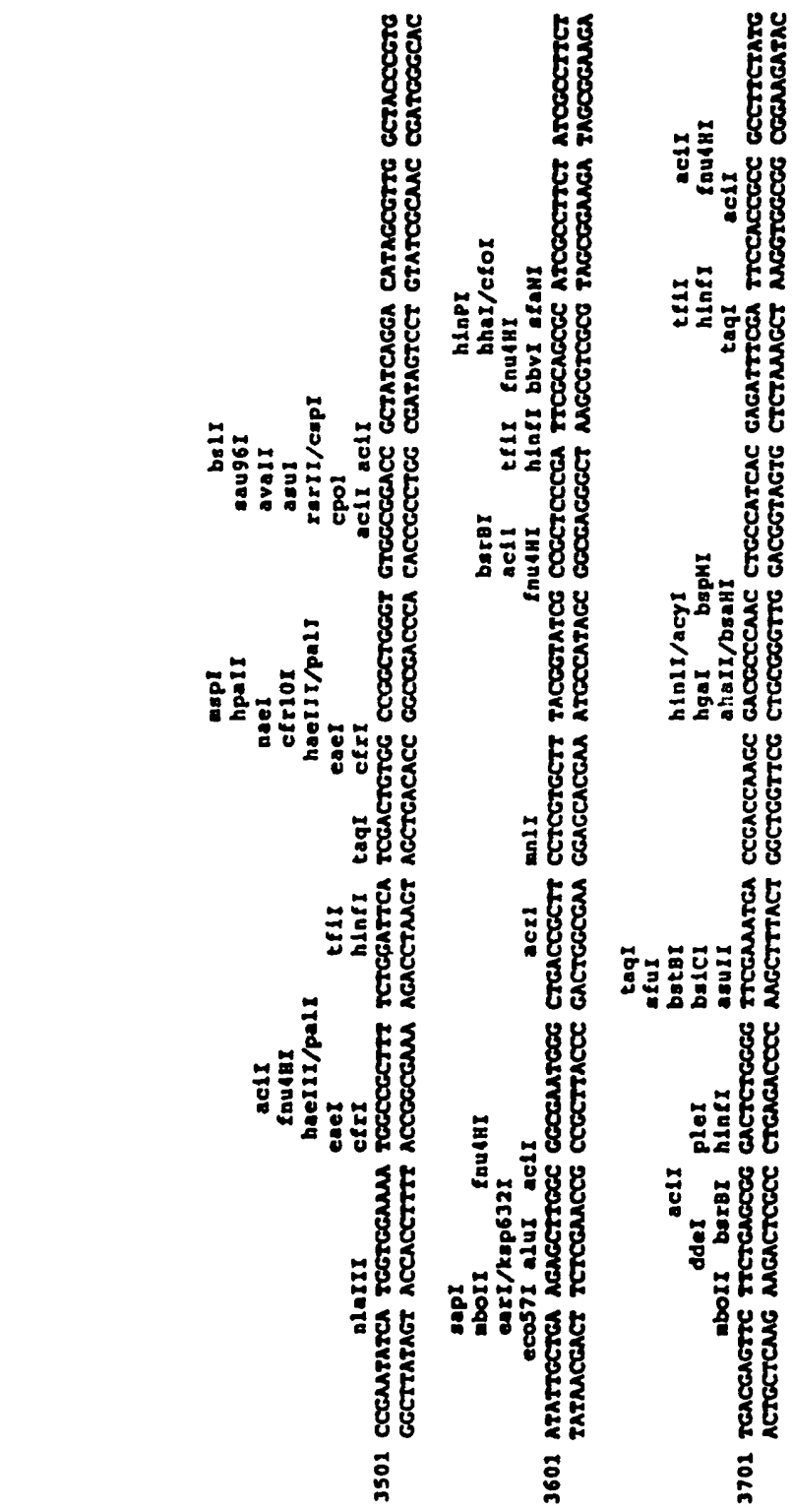

aspI bslI
hpaII sau96I
naeI avaII
cfr10I asuI
haeIII/paII rsrII/csp
caeI cpoI
taqI cfrI acII acII
3501 CCGAATATCA TGGTGGAAAA TGCCCCCTTT TCTGGATTCA TCGACTGTGG CCGCCTCGGT GTGGCGGACC GCTATCAGGA CATAGCGTTG GCTACCCGTG
GGCTTATAGT ACCACCTTTT ACGGGCGAAA AGACCTAAGT AGCTGACACC GGCGGACCCA CACCGCCTGG CGATAGTCCT GTATCGCAAC CGATGGGCAC
hinPI
bsrBI bbaI/cfoI
acII tfiI fnu4HI
fnu4HI hinfI bbvI sfaNI
sapI
aboII fnu4HI
earI/ksp632I
eco57I aluI acII acII mnlI
3601 ATATTGCTGA AGAGCTTGGC GGCGAATGGG CTGACCGCTT CCTCGTGCTT TACGGTATCG CCGCTCCCGA TTCGCAGCGC ATCGCCTTCT ATCGCCTTCT
TATAACGACT TCTCGAACCG CCGCTTACCC GACTGGCGAA GGAGCACGAA ATGCCATAGC GGCGAGGGCT AAGCGTCGCG TAGCGGAAGA TAGCGGAAGA
taqI
sfuI
bstBI
acII hinII/acyI tfiI acII
ddeI pleI bsiCI hgaI bspMI hinfI fnu4HI
aboII bsrBI hinfI asuII ahaII/bsaHI taqI acII
3701 TCACCAGTTC TTCTGAGCGG GACTCTGGGG TTCGAAATGA CCGACCAAGC GACGCCCAAC CTGCCATCAC GAGATTTCGA TTCCACCGCC GCCTTCTATG
ACTGGTCAAG AAGACTCCCC CTGAGACCCC AAGCTTTACT GGCTGGTTCG CTGCGGGTTG GACGGTAGTG CTCTAAAGCT AAGGTGGCGG CGGAAGATAC

FIG. 6M

```
                                                                                                    scrFI
                                                                                                    ncII
                                                                                                    mspI
                                                                                                    hpaII
                                                                                                    dsaV
                                                                                                    cauII
                                                                                                    bslI
                                    hinlI/acyI                 thaI  sau3AI                        xmaI/pspAI
                                    hgaI              bslI fnuDII/mvnI                             smaI
                                    ahaII/bsaHI         gsuI/bpmI   bstYI/xhoII                    scrFI
                                ncII               mnlI   hinPI alwI[dam-]                         ncII
                                scrFI               sau3AI    hhaI/cfoI        gsuI/bpmI           dsaV
                                mspI     mspI       mboI/ndeII[dam-] mboI/ndeII[dam-]              cauII
                                hpaII    hpaII      dpnI[dam+] bstUI dpnI[dam+]                    bsaJI
                     tfiI       dsaV    naeI        dpnII[dam-] aciI dpnII[dam-]                  bsaJI
                     hinfI      cauII   cfr10I fokI alwI[dam-] bsh1236I    nlaIII      mboII  bslI avaI
3801 AAAGGTTGGG CTTCGGAATC GTTTTCCGGG ACGCCGGCTG GATGATCCTC CAGCGCGGGG ATCTCATGCT GGAGTTCTTC GCCCACCCCG GGAGATGGGG
     TTTCCAACCC GAAGCCTTAG CAAAAGGCCC TGCGGCCGAC CTACTAGGAG GTCGCGCCCC TAGAGTACGA CCTCAAGAAG CGGGTGGGGC CCTCTACCCC
                                                                           HSV1 tk terminator SmaI-PvuII  ^

                                                     hinPI
                                                     hhaI/cfoI
                                                    thaI
                                                    fnuDII/mvnI
                                      mspI          bstUI
                                      hpaII         bsh1236I
     mnlI                    bsmAI    bsaWI  nlaIV aciI
3901 GAGGCTAACT GAAACACGGA AGGAGACAAT ACCGGAAGGA ACCCGCGCTA TGACGGCAAT AAAAAGACAG AATAAAACGC ACGGGTGTTG GGTCGTTTGT
     CTCCGATTGA CTTTGTGCCT TCCTCTGTTA TGGCCTTCCT TGGGCGCGAT ACTGCCGTTA TTTTTCTGTC TTATTTTGCG TGCCCACAAC CCAGCAAACA

                            scrFI
                            mvaI
                            ecoRII
                            dsaV
                            bstNI
                            bsaJI
                           bslI
             aciI      sau96I                                                           thaI
            thaI       nlaIV                                               haeIII/palI  fnuDII/mvnI
            fnuDII/mvnI    bsaJI                                 bslI     sau96I        bstUI
            bstUI      avaII                                 bsmAI        asuI          bsh1236I
            bsh1236I   asuI apyI[dcm+]       taqI            bsaI        nlaIV         aciI    mboII
4001 TCATAAACGC GGGGTTCGGT CCCAGGGCTG GCACTCTGTC GATACCCCAC CGAGACCCCA TTGGGGCCAA TACGCCCGCG TTTCTTCCTT TTCCCCACCC
     AGTATTTGCG CCCCAAGCCA GGGTCCCGAC CGTGAGACAG CTATGGGGTG GCTCTGGGGT AACCCCGGTT ATGCGGGCGC AAAGAAGGAA AAGGGGTGGG
```

FIG. 6N

hgiJII
bsp1286
bayI
scrFI
avaI
ecoRII
dsaV
bstNI
bsaJI
apyI(dcm+)
bsaJI
sau96I bslI
bslI haeIII/paII fnu4HI
bslI bphI asuI banII bbvI
4101 CAACCCCCAA GTTCGGGTCA AGGCCCAGGG CTCGCAGCCA ACGTCGGGGC GGCAAGCCCG CCATAGCCAC GGGCCCCGTG GGTTAGGGAC GGGGTCCCCC
GTTGGGGGTT CAAGCCCACT TCCGGGTCCC GAGCGTCGGT TGCAGCCCCG CCGTTCGGGC GGTATCGGTG CCCGGGGCAC CCAATCCCTG CCCCAGGGGG
xcmI asuI dsaI
dsaI apaI bsaJI
bsaJI asuI
nlaIII
styI
ncoI
dsaI
bsaJI
haeIII/paII
baeI
fokI
4201 ATGCGCAATG GTTTATCGTT CGTGGGGGTT ATTCTTTTGG GCGTTGCGTG GGGTCAGGTC CACGACTGGA CTGAGCAGAC AGACCCATGG TTTTTGGATG
TACGCGTTAC CAAATAGCAA GCACCCCCAA TAAGAAAACC CGCAACGCAC CCCAGTCCAG GTGCTGACCT GACTCGTCTG TCTGGGTACC AAAAACCTAC
scrFI
avaI
ecoRII
dsaV
bstNI
bslI
bsaJI nlaIII
apyI(dcm+)
4301 GCCTGGGCAT GGACCGCATC TACTGGCGCG ACACGAACAC CGGCCGTCTG TGGCTGCCAA ACACCCCCGA CCCCCAAAAA CCACCGCGCC GATTTCTGGC
CGGACCCGTA CCTGGCGTAG ATGACCGCGC TGTGCTTGTG GCCCGCAGAC ACCGACGGTT TGTGGGGGCT GGGGTTTTTT GGTGGCGCGG CTAAAGACCG
hinII/acyI
ahaII/bsaHI
scrFI
nciI
hpaII
dsaV hgaI
cauII
fnu4HI
bbvI
aclI
thaI
fnuDII/mvnI hinPI
bstUI hhaI/cfoI
bsh1236I nlaIV narI
hinPI kasI
thaI fnuDII/mvnI hinlI/acyI
bstUI hgiCI
bsh1236I haeII
aclI banI
bslI hhaI/cfoI ahaII/bsaHI

FIG. 6O

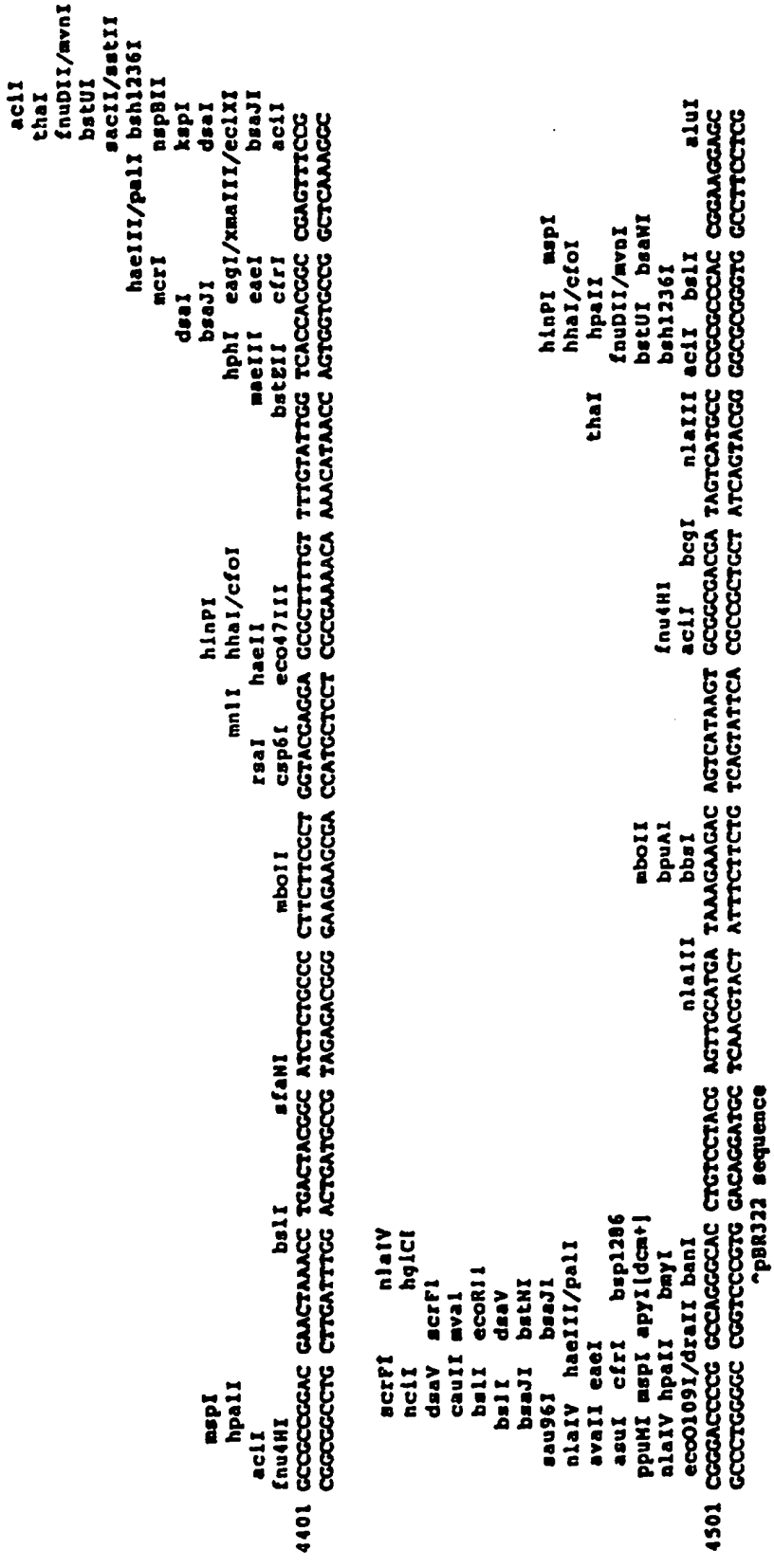

aclI
thaI
fnuDII/mvnI
bstUI
sacII/sstII
haeIII/palI bsh1236I
ncrI nspBII
dsaI kspI
bsaJI dsaI
hinPI
mnlI hhaI/cfoI hphI eagI/xmaIII/eclXI
rsaI haeII maeIII eaeI bsaJI
csp6I eco47III bstEII cfrI aclI
mboII
sfaNI bslI
4401 GCCGCCGGAC GAACTAAACC TGACTACGGC ATCTCTGCCC CTTCTTCCCT CGTACGAGGA GCGCTTTTGT TTTGTATTGG TCACCACGGC CGAGTTTCCG
CGGCGGCCTG CTTGATTTGG ACTGATGCCG TAGAGACGGG GAAGAAGGGA CCATGCTCCT CGCGAAAACA AAACATAACC AGTGGTGCCG GCTCAAAGGC
hinPI mspI
hhaI/cfoI
thaI hpaII
fnuDII/mvnI
bstUI bsaWI
bsh1236I
nlaIII aclI bslI alwI
fnu4HI
aclI bcgI
mboII
bpuAI
bbsI
nlaIII
scrFI nlaIV
nciI hgiCI
dsaV scrFI
cauII mva1
bslI ecoRII
bslI dsaV
bsaJI bstNI
sau96I bsaJI
nlaIV haeIII/palI
mva1I eaeI
asuI cfrI bsp1286
ppuMI mspI apyI[dcm+]
nlaIV hpaII bmyI
eco0109I/draII banI
4501 CGGGACCCCG GCCAGGGCAC CTGTCCTACG AGTTGCATGA TAAAGAGAC AGTCATAAGT GCGGCGACGA TAGTCATGCC CCGCGCCCAC CGGAAGGAGC
GCCCTGGGGC CGGTCCCGTG GACAGGATGC TCAACGTACT ATTTCTTCTG TCAGTATTCA CGCCGCTGCT ATCAGTACGG GGCGCGGGTG GCCTTCCTCG
^pBR322 sequence

FIG. 6P

```
                                              fnu4HI
                                             haeIII/palI                                              aciI
                                            mcrI                                                  fnu4HI
                                            eagI/xmaIII/eclXI            hinPI                   thaI
                                            eaeI                        thaI                     fnuDII/mvnI
                                           notI                         fnuDII/mvnI              bstUI
                                           fnu4HI                       bstUI scfI     hinPI    hinPI
                                           aciI                         bsh1236I       hhaI/cfoI hhaI/cfoI
                                   mcrI  bsrBI aciI                 rsaI hhaI/cfoI  fnu4HI   tru9I aciI
       bsrI                    sfaNI   taqI cfrI  sfaNI             csp6I   bslI    aciI     mseI bsh1236I
4601 TGACTGGGTT GAAGGCTCTC AAGGGCATCG GTCGAGCGGC CGCATCAAAG CAACCATAGT ACGCGCCCTG TAGCGGCGCA TTAAGCGCGG CGGGTGTGGT
     ACTGACCCAA CTTCCGAGAG TTCCCGTAGC CAGCTCGCCG GCGTAGTTTC GTTGGTATCA TGCGCGGGAC ATCGCCGCGT AATTCGCGCC GCCCACACCA
                                                   ^delta 3
                                                    ^M13 ori

             fnu4HI
           hinPI
           hhaI/cfoI                                hinPI
          thaI                                      hhaI/cfoI
          fnuDII/mvnI                           rmaI
          bstUI                            hinPI  haeII                                                mspI
          bsh1236I    aciI                 hhaI/cfoI  bsrBI                                            hpaII
      maeIII bbvI  maeIII                 haeII maeI  aciI             mboII                  maeII  naeI
                                                                                                     cfr10I
4701 GGTTACGCGC AGCGTGACCG CTACACTTGC CAGCGCCCTA GCGCCCGCTC CTTTCGCTTT CTTCCCTTCC TTTCTCGCCA CGTTCGCCGG CTTTCCCCGT
     CCAATGCGCG TCGCACTGGC GATGTGAACG GTCGCGGGAT CGCGGGCGAG GAAAGCGAAA GAAGGGAAGG AAAGAGCGGT GCAAGCGGCC GAAAGGGGCA

                      nlaIV
                     hgiJII
                     bsp1286                                  nlaIV                                maeII    haeIII/palI
                     bmyI                                     hgiCI  taqI                         draIII   sau96I
       aluI          banII          nlaIV                     banI mnlI                  hphI     bsaAI    asuI
4801 CAAGCTCTAA ATCGGGGGCT CCCTTTAGGG TTCCGATTTA GTGCTTTACG GCACCTCGAC CCCAAAAAAC TTGATTTGGG TGATGGTTCA CGTAGTGGGC
     GTTCGAGATT TAGCCCCCGA GGGAAATCCC AAGGCTAAAT CACGAAATGC CGTGGAGCTG GGGTTTTTTG AACTAAACCC ACTACCAAGT GCATCACCCG

                                       maeII pleI          tru9I     pleI                             bslI
                                      drdI  hinfI  maeII   mseI      hinfI       bsrI                bslI  avaI
4901 CATCGCCCTG ATAGACGGTT TTTCGCCCTT TGACGTTGGA GTCCACGTTC TTTAATAGTG GACTCTTGTT CCAAACTGGA ACAACACTCA ACCCTATCTC
     GTAGCGGGAC TATCTGCCAA AAAGCGGGAA ACTGCAACCT CAGGTGCAAG AAATTATCAC CTGAGAACAA GGTTTGACCT TGTTGTGAGT TGGGATAGAG
```

FIG. 6Q


thaI
fnuDII/mvnI
tru9I
mseI
tru9I
tru9I apoI tru9I
mseI bstUI mseI
haeIII/pallI
aluI
mseI
apoI
bsh1236I
sspI
5001 GGGCTATTCT TTTGATTTAT AAGGGATTTT GCCGATTTCG GCCTATTGGT TAAAAAATGA GCTGATTTAA CAAAAATTTA ACGCGAATTT TAACAAAATA
CCCGATAAGA AAACTAAATA TTCCCTAAAA CGGCTAAAGC CGGATAACCA ATTTTTTACT CGACTAAATT GTTTTTAAAT TGCGCTTAAA ATTGTTTTAT
maeII
psp1406I
tru9I
mseI
mnlI
haeIII/pallI
stuI
haeI
nlaIII
tru9I rcaI
mseI bspHI
maeII
hinII/acyI
ahaII/bsaHI
ddeI aatII
5101 TTAACGTTTA CAATTTTATG GTGCAGGCCT CGTGATACGC CTATTTTTAT AGGTTAATGT CATGATAATA ATGGTTTCTT AGACGTCAGG TGGCACTTTT
AATTGCAAAT GTTAAAATAC CACGTCCGGA GCACTATGCG GATAAAAATA TCCAATTACA GTACTATTAT TACCAAAGAA TCTGCAGTCC ACCGTGAAAA
^delta 2a
nlaIV
aciI
thaI
fnuDII/mvnI
bstUI
bsh1236I
hinPI
hhaI/cfoI
rcaI
bspHI
bsrBI bsmAI
aciI nlaIII
sspI
5201 CGGGGAAATG TGCGCGGAAC CCCTATTTGT TTATTTTTCT AAATACATTC AAATATGTAT CCGCTCATGA GACAATAACC CTGATAAATG CTTCAATAAT
GCCCCTTTAC ACGCGCCTTG GGGATAAACA AATAAAAAGA TTTATGTAAG TTTATACATA GGCGAGTACT CTGTTATTGG GACTATTTAC GAAGTTATTA
mboII
earI/ksp632I
fnu4HI
aciI
hphI
5301 ATTGAAAAAG GAAGAGTATG AGTATTCAAC ATTTCCGTGT CGCCCTTATT CCCTTTTTTG CGGCATTTTG CCTTCCTGTT TTTGCTCACC CAGAAACGCT
TAACTTTTTC CTTCTCATAC TCATAAGTTG TAAAGGCACA GCGGGAATAA GGGAAAAAAC GCCGTAAAAC GGAAGGACAA AAACGAGTGG GTCTTTGCGA
hgiAI/aspHI
bsp1286
sau3AI
bsiHKAI
sau3AI
mboI/ndeII[dam-]
dpnI[dam+]
dpnII[dam-]
bmyI
sau3AI
mboI/ndeII[dam-]
dpnI[dam+]
dpnII[dam-]
bstYI/xhoII
sau3AI
mboI/ndeII[dam-]
dpnI[dam+]
dpnII[dam-]
alwI[dam-]
eco57I
apaLI/snoI
bsrI
nspBII
hphI
sfaNI mboII[dam-]
alw44I/snoI
maeIII taqI
alwI[dam-] aciI
bstYI/xhoII
mboII
5401 GGTGAAAGTA AAAGATGCTG AAGATCAGTT GGGTGCACGA GTGGGTTACA TCGAACTGGA TCTCAACAGC GGTAAGATCC TTGAGAGTTT TCGCCCCGAA
CCACTTTCAT TTTCTACGAC TTCTAGTCAA CCCACGTGCT CACCCAATGT AGCTTGACCT AGAGTTGTCG CCATTCTAGG AACTCTCAAA AGCGGGGCTT

FIG. 6R

```
                                                                              scrFI
                                                                              ncII
                                                      aciI                    mspI
                                                     thaI                     hpaII
                                                     fnuDII/mvnI              dsaV
       maeII           hgiAI/aspHI                   bstUI                    cauII
      psp1406I         bsp1286  tru9I                bsh1236I             hinlI/acyI
     xmnI              bsiHKAI  mseI                hinPI                 hgaI                        aciI
     asp700            bmyI    ahaIII/draI          hhaI/cfoI             ahaII/bsaHI    bcgI   mcrI fnu4HI
5501 GAACGTTTTC CAATGATGAG CACTTTTAAA GTTCTGCTAT GTGGCGCGGT ATTATCCCGT GATGACGCCG GGCAAGAGCA ACTCGGTCGC CGCATACACT
     CTTGCAAAAG GTTACTACTC GTGAAAATTT CAAGACGATA CACCGCGCCA TAATAGGGCA CTACTGCGGC CCGTTCTCGT TGAGCCAGCG GCGTATGTGA

                             rsaI
                             csp6I  bsrI                                                        fnu4HI
        ddeI                scaI hphI   maeIII      sfaNI     fokI  nlaIII                      bbvI        nlaIII
5601 ATTCTCAGAA TGACTTGGTT GAGTACTCAC CAGTCACAGA AAAGCATCTT ACGGATGGCA TGACAGTAAG AGAATTATGC AGTGCTGCCA TAACCATGAG
     TAAGAGTCTT ACTGAACCAA CTCATGAGTG GTCAGTGTCT TTTCGTAGAA TGCCTACCGT ACTGTCATTC TCTTAATACG TCACGACGGT ATTGGTACTC

                                                 sau96I
                                                 avaII
                                         sau3AI  asuI                                         nlaIII
                  haeIII/palI            mboI/ndeII(dam-)                                 sau3AI maeIII
                 eaeI                    dpnI(dam+)                                       mboI/ndeII(dam-)    sau3AI
                 cfrI                    dpnII(dam-)                                      dpnI(dam+)          mboI/ndeII(dam-)
                fnu4HI                  pvuI/bspCI                                       alwI(dam-)           dpnI(dam+)
                aciI                    mcrI  mnlI          aluI  aciI              nlaIII dpnII(dam-)         dpnII(dam-)
5701 TGATAACACT GCGGCCAACT TACTTCTGAC AACGATCGGA GGACCGAAGG AGCTAACCGC TTTTTTGCAC AACATGGGGG ATCATGTAAC TCGCCTTGAT
     ACTATTGTGA CGCCGGTTGA ATGAAGACTG TTGCTAGCCT CCTGGCTTCC TCGATTGGCG AAAAAACGTG TTGTACCCCC TAGTACATTG AGCGGAACTA

                                                                                             hinPI
              mspI                                                                         mstI
              hpaII                                                                        aviII/fspI      bsrI
             bsaWI                                                     fnu4HI          maeII hhaI/cfoI  tru9I
          nlaIV    aluI                             maeIII     sfaNI   bbvI           psp1406I          mseI
5801 CGTTGGGAAC CGGAGCTGAA TGAAGCCATA CCAAACGACG AGCGTGACAC CACGATGCCA GCAGCAATGG CAACAACGTT GCGCAAACTA TTAACTGGCG
     GCAACCCTTG GCCTCGACTT ACTTCGGTAT GGTTTGCTGC TCGCACTGTG GTGCTACGGT CGTCGTTACC GTTGTTGCAA CGCGTTTGAT AATTGACCGC
```

FIG. 6S


5901 AACTACTTAC TCTAGCTTCC CGGCAACAAT TAATAGACTG GATGGAGGCG GATAAAGTTG CAGGACCACT TCTGCGCTCG GCCCTTCCGG CTGGCTGGTT
TTGATGAATG AGATCGAAGG GCCGTTGTTA ATTATCTGAC CTACCTCCGC CTATTTCAAC GTCCTGGTGA AGACGCGAGC CGGGAAGGCC GACCGACCAA
6001 TATTGCTGAT AAATCTGGAG CCGGTGAGCG TGGGTCTCGC GGTATCATTG CAGCACTGGG GCCAGATGGT AAGCCCTCCC GTATCGTAGT TATCTACACG
ATAACGACTA TTTAGACCTC GGCCACTCGC ACCCAGAGCG CCATAGTAAC GTCGTGACCC CGGTCTACCA TTCGGGAGGG CATAGCATCA ATAGATGTGC
6101 ACGGGCAGTC AGGCAACTAT GGATGAACGA AATAGACAGA TCGCTGAGAT AGGTGCCTCA CTGATTAAGC ATTGGTAACT GTCAGACCAA GTTTACTCAT
TGCCCCTCAG TCCGTTGATA CCTACTTGCT TTATCTGTCT AGCGACTCTA TCCACGGAGT GACTAATTCG TAACCATTGA CAGTCTGGTT CAAATGAGTA
6201 ATATACTTTA GATTGATTTA AAACTTCATT TTTAATTTAA AAGGATCTAG GTGAAGATCC TTTTTGATAA TCTCATGACC AAAATCCCTT AACGTGAGTT
TATATGAAAT CTAACTAAAT TTTGAAGTAA AAATTAAATT TTCCTAGATC CACTTCTAGG AAAAACTATT AGAGTACTGG TTTTAGGGAA TTGCACTCAA

FIG. 6T

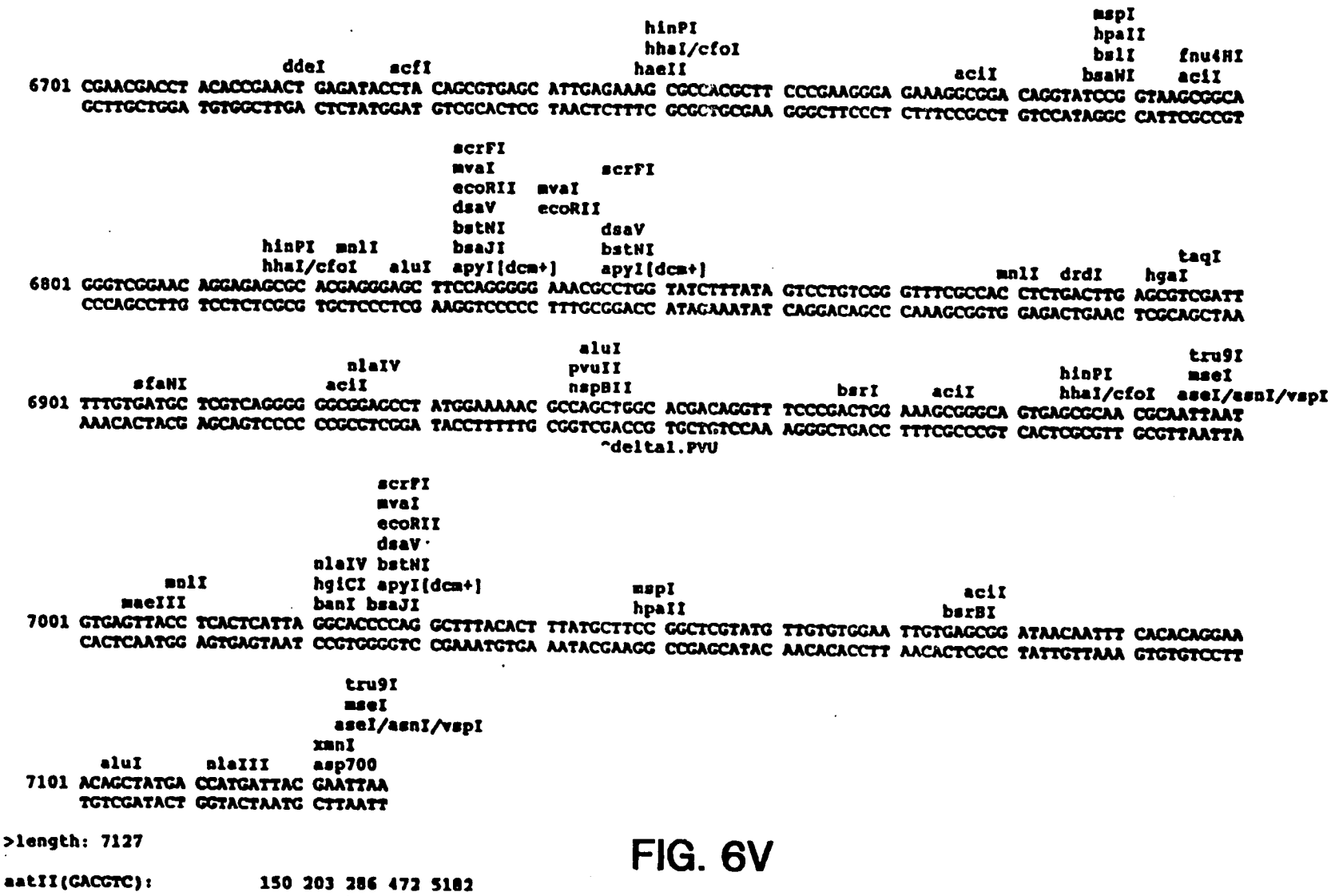
```
                                             sau3AI
                                             mboI/ndeII(dam-)
                                             dpnI(dam+)  sau3AI            thaI
                                             dpnII(dam-) mboI/ndeII(dam-)
                                            bstYI/xhoII  dpnI(dam+)        fnuDII/mvnI
                                   sau3AI   alwI(dam-)   dpnII(dam-)       bstUI
                                   mboI/ndeII(dam-)      alwI(dam-)        bsh1236I
                                   dpnI(dam+) mboII(dam-)                 hinPI       fnu4HI
            ddeI hgaI              dpnII(dam-)          bstYI/xhoII       hhaI/cfoI   bbvI
6301 TTCGTTCCAC TGAGCGTCAG ACCCCGTAGA AAAGATCAAA GGATCTTCTT GAGATCCTTT TTTTCTGCGC GTAATCTGCT GCTTGCAAAC AAAAAAACCA
     AAGCAAGGTG ACTCGCAGTC TGGGGCATCT TTTCTAGTTT CCTAGAAGAA CTCTAGGAAA AAAAGACGCG CATTAGACGA CGAACGTTTG TTTTTTTGGT

                              sau3AI
                              mboI/ndeII(dam-)
                              dpnI(dam+)
                              dpnII(dam-)
                             alwI(dam-)
            aciI             mspI                                     bsrI                 hinPI                  rmaI
     aciI  nspBII            hpaII        aluI                     maeIII    eco57I        hhaI/cfoI              maeI
6401 CCGCTACCAG CGGTGGTTTG TTTGCCGGAT CAAGAGCTAC CAACTCTTTT TCCGAAGGTA ACTGGCTTCA GCAGAGCGCA GATACCAAAT ACTGTCCTTC
     GGCGATGGTC GCCACCAAAC AAACGGCCTA GTTCTCGATG GTTGAGAAAA AGGCTTCCAT TGACCGAAGT CGTCTCGCGT CTATGGTTTA TGACAGGAAG

                                                                                                  fnu4HI
                                                                                    alwNI      bbvI
                 haeIII/palI                                                       bsrI     fnu4HI
          bslI    haeI               scfI      aciI        mnlI                 maeIII      bbvI     bsrI
6501 TAGTGTAGCC GTAGTTAGGC CACCACTTCA AGAACTCTGT AGCACCGCCT ACATACCTCG CTCTGCTAAT CCTGTTACCA GTGGCTGCTG CCAGTGGCGA
     ATCACATCGG CATCAATCCG GTGGTGAAGT TCTTGAGACA TCGTGGCGGA TGTATGGAGC GAGACGATTA GGACAATGGT CACCGACGAC GGTCACCGCT

                scrFI                                         mcrI                          hgiAI/aspHI
                ncII                                        nspBII                          bsp1286
                mspI                                 mspI  fnu4HI                           bsiHKAI
                hpaII                                hpaII bbvI                             bmyI
                dsaV     pleI                      bsaWI  hinPI aciI                        apaLI/snoI
                cauII    hinfI                  maeIII    hhaI/cfoI                         alw44I/snoI   aluI
6601 TAAGTCGTGT CTTACCGGGT TGGACTCAAG ACGATAGTTA CCGGATAAGG CGCAGCGGTC GGGCTGAACG GGGGGTTCGT GCACACAGCC CAGCTTGGAG
     ATTCAGCACA GAATGGCCCA ACCTGAGTTC TGCTATCAAT GGCCTATTCC GCGTCGCCAG CCCGACTTGC CCCCCAAGCA CGTGTGTCGG GTCGAACCTC
```

FIG. 6U



>length: 7127

aatII(GACGTC): 150 203 286 472 5182

FIG. 6V

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 96/20718

A. CLASSIFICATI N OF SUBJECT MATTER
IPC 6 C12N15/16 C07K14/575 A61K38/22 C12N15/70 C12N1/21
//(C12N1/21,C12R1:19)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
E	WO 97 00319 A (SMITHKLINE BEECHAM PLC ;BROWNE MICHAEL JOSEPH (GB); CHAPMAN CONRAD) 3 January 1997 see page 1, line 31 - line 33; claims; examples ---	1-4,7-26
P,X	EP 0 741 187 A (HOFFMANN LA ROCHE) 6 November 1996 see page 9, line 19 - page 11, line 46; claims; examples 19,20 ---	1-3, 6-12,26
P,X	WO 96 05309 A (UNIV ROCKEFELLER ;FRIEDMAN JEFFREY M (US); ZHANG YIYING (US); PROE) 22 February 1996 see page 43, line 3 - page 46, line 14; claims --- -/--	1-3, 6-12,26

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
17 April 1997	14.05.97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Fuhr, C

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 96/20718

C.(Continuation) D CUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	NATURE, vol. 372, no. 6505, 1 December 1994, pages 425-432, XP000602062 YIYING ZHANG ET AL: "POSITIONAL CLONING OF THE MOUSE OBESE GENE AND ITS HUMAN HOMOLOGUE" see the whole document -----	1,10-12, 26

1

INTERNATIONAL SEARCH REPORT

:ernational application No.
PCT/US 96/ 20718

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 7-10,24-25
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 7-10, and 24-25 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Inter ional Application No
PCT/US 96/20718

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9700319 A	03-01-97	AU 6011096 A	15-01-97
EP 0741187 A	06-11-96	AU 5197896 A	14-11-96
		CA 2175298 A	06-11-96
		ES 2093593 T	01-01-97
		JP 9003098 A	07-01-97
		NO 961796 A	06-11-96
		PL 314051 A	12-11-96
WO 9605309 A	22-02-96	AU 3329895 A	07-03-96
		CA 2195955 A	22-02-96
		DE 19531931 A	07-03-96
		FI 970656 A	17-02-97
		GB 2292382 A	21-02-96
		JP 9502729 T	18-03-97
		ZA 9506868 A	09-04-96